

FORM PTO-1390 (Modified)  
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

ATTORNEY'S DOCKET NUMBER

**217865US0JPCT**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

**10/031569**INTERNATIONAL APPLICATION NO.  
**PCT/JP00/04843**INTERNATIONAL FILING DATE  
**19 JULY 2000**PRIORITY DATE CLAIMED  
**21 JULY 1999 (EARLIEST)**

## TITLE OF INVENTION

**CHOLESTEROL-LOWERING AGENTS, SECONDARY BILE ACID PRODUCTION INHIBITORS AND FOODS AND DRINKS**

## APPLICANT(S) FOR DO/EO/US

**Haruji SAWADA, et al.**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1.  This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2.  This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3.  This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4.  The US has been elected by the expiration of 19 months from the priority date (Article 31).
5.  A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a.  is attached hereto (required only if not communicated by the International Bureau).
  - b.  has been communicated by the International Bureau.
  - c.  is not required, as the application was filed in the United States Receiving Office (RO/US).
6.  An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a.  is attached hereto.
  - b.  has been previously submitted under 35 U.S.C. 154(d)(4).
7.  Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a.  are attached hereto (required only if not communicated by the International Bureau).
  - b.  have been communicated by the International Bureau.
  - c.  have not been made; however, the time limit for making such amendments has NOT expired.
  - d.  have not been made and will not be made.
8.  An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9.  An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10.  An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11.  A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12.  A copy of the International Search Report (PCT/ISA/210).

**Items 13 to 20 below concern document(s) or information included:**

13.  An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14.  An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15.  A **FIRST** preliminary amendment.
16.  A **SECOND** or **SUBSEQUENT** preliminary amendment.
17.  A substitute specification.
18.  A change of power of attorney and/or address letter.
19.  A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20.  A second copy of the published international application under 35 U.S.C. 154(d)(4).
21.  A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22.  Certificate of Mailing by Express Mail
23.  Other items or information:

Notice of Priority / PCT/IB/304 / PCT/IB/308

PTO-1449 / Drawing (1 sheet)

Amended Sheets (66, 67, 68 and 69)

| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR<br><b>10/031569</b>  | INTERNATIONAL APPLICATION NO.<br><b>PCT/JP00/04843</b> | ATTORNEY'S DOCKET NUMBER<br><b>217865US0JPCT</b>                              |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
|---|--|---|---|--------------|------|--------------|-----------|---|--------------------------|--------------------|---------|---|---------------------------|--|--|--|---|--|
| 24. The following fees are submitted:   |  | <b>CALCULATIONS PTO USE ONLY</b>  |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</b>  |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1040.00</b><br><input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890.00</b><br><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740.00</b><br><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710.00</b><br><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b> |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>   |  | <b>\$890.00</b>   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).   |  | <input type="checkbox"/> 20 <input type="checkbox"/> 30 <b>\$0.00</b>         |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 25%;">CLAIMS</th> <th style="width: 25%;">NUMBER FILED</th> <th style="width: 25%;">NUMBER EXTRA</th> <th style="width: 25%;">RATE</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>25 - 20 =</td> <td>5</td> <td>x \$18.00    <b>\$90.00</b></td> </tr> <tr> <td>Independent claims</td> <td>9 - 3 =</td> <td>6</td> <td>x \$84.00    <b>\$504.00</b></td> </tr> <tr> <td colspan="3">Multiple Dependent Claims (check if applicable).</td> <td style="text-align: center;"><input checked="" type="checkbox"/>    <b>\$280.00</b></td> </tr> </tbody> </table>  |  | CLAIMS  | NUMBER FILED  | NUMBER EXTRA | RATE | Total claims | 25 - 20 = | 5 | x \$18.00 <b>\$90.00</b> | Independent claims | 9 - 3 = | 6 | x \$84.00 <b>\$504.00</b> | Multiple Dependent Claims (check if applicable). |  |  | <input checked="" type="checkbox"/> <b>\$280.00</b> |  |
| CLAIMS  | NUMBER FILED   | NUMBER EXTRA  | RATE  |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| Total claims  | 25 - 20 =  | 5   | x \$18.00 <b>\$90.00</b>                            |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| Independent claims  | 9 - 3 =  | 6   | x \$84.00 <b>\$504.00</b>                           |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| Multiple Dependent Claims (check if applicable).  |  |   | <input checked="" type="checkbox"/> <b>\$280.00</b> |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>TOTAL OF ABOVE CALCULATIONS =</b>  |  | <b>\$1,764.00</b>   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.   |  | <b>\$0.00</b>   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>SUBTOTAL =</b>   |  | <b>\$1,764.00</b>   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| Processing fee of <b>\$130.00</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).  |  | <input type="checkbox"/> 20 <input type="checkbox"/> 30    + <b>\$0.00</b>    |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>TOTAL NATIONAL FEE =</b>   |  | <b>\$1,764.00</b>   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).   |  | <input type="checkbox"/> <b>\$0.00</b>  |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>TOTAL FEES ENCLOSED =</b>  |  | <b>\$1,764.00</b>   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
|   |  | <b>Amount to be:</b><br><b>refunded</b> <b>\$</b><br><b>charged</b> <b>\$</b> |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| a. <input checked="" type="checkbox"/> A check in the amount of <b>\$1,764.00</b> to cover the above fees is enclosed.<br>b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.<br>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <b>15-0030</b> A duplicate copy of this sheet is enclosed.<br>d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING: Information on this form may become public. Credit card information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| SEND ALL CORRESPONDENCE TO:   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <br><b>22850</b>   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>Surinder Sachar</b><br><b>Registration No. 34,423</b>  |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <i>Surinder Sachar</i><br><b>SIGNATURE</b>  |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>Norman F. Oblon</b><br><b>NAME</b>   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <b>24,618</b><br><b>REGISTRATION NUMBER</b>   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| <i>Jan 22 2002</i><br><b>DATE</b>   |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |
| (703) 413-3000  |  |   |   |              |      |              |           |   |                          |                    |         |   |                           |  |  |  |   |  |

217865US

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF : :

HARUJI SAWADA ET AL. : :

SERIAL NO: NEW APPLN. : ATTN: APPLICATION BRANCH  
(Based on PCT/JP00/4843)

FILED: HEREWITH : :

FOR: CHOLESTEROL-LOWERING AGENTS, SECONDARY BILE ACID  
PRODUCTION INHIBITORS AND FOODS AND DRINKS

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

At page 70, (Abstract), after the last line, beginning on the next page, please insert Figure 1 attached hereto on a separate piece of paper.

REMARKS

Claims 1-19 are active in the present application.

Applicants have submitted Figure 1 in the form of drawings. Support for Figure 1 is found at page 10, lines 2-5, and from page 29, line 19 to page 30, line 10. No new matter is believed to be introduced by the submission of Figure 1.

Applicants submit that this application is ready for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.



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1/Prds

Description

Cholesterol-Lowering Agents, Secondary Bile Acid Production Inhibitors, and Foods and Drinks

Technical Field

The present invention relates to a cholesterol-lowering agent capable of lowering the blood or hepatic cholesterol level and improving atherogenic index; to a secondary bile acid production inhibitor useful for preventing and treating colorectal cancer, liver cancer, pancreatic cancer, cholelithiasis, etc.; and to use thereof for producing foods and drinks.

Background Art

In recent years, the mortality rate relating to cardiovascular diseases has increased year by year. If diseases caused by arteriosclerosis such as cardiac infarction and brain infarction are included, cardiovascular diseases are ranked at the top of the causes of death in adults.

Arteriosclerosis is caused by a variety of factors. Among them, increase in plasma lipid level, *inter alia*, plasma cholesterol level, is conceivably one of the most dangerous factors.

One cause of elevated plasma cholesterol level is a genetic disease. In this case, grave patients are given diet

therapy, and simultaneously, drugs such as a cholesterol synthesis inhibitor, Nicomol, Clofibrate, an ion-exchange resin, and an anabolic steroid are employed. However, these drugs cause adverse side effects such as toxicity to the liver, gastrointestinal disorders, and carcinogenicity.

Another important cause of elevated plasma cholesterol level is excessive fat consumption caused by the recent dietary custom of consuming a large quantity of eggs, butter, meat, etc., to the extent that excessive fat consumption has become the general habit for younger people. Alimentary hypercholesterolemia induced by excessive fat consumption generally does not become grave in comparison with that caused by genetic factor. But alimentary hypercholesterolemia induces a gradual accumulation of cholesterol in the vascular wall from a young age and problematically causes arteriosclerosis in adulthood, and then may cause cardiac infarction and brain infarction together with hypertriglyceridemia. In relation to these hyperlipemias, diet therapy including limitation of lipid consumption within an appropriate range is of great importance, rather than drug therapy, which raises problems such as adverse side effects. However, patients under diet limitation suffer mental pain and must abandon the joy of their regular diet. Thus, complete diet therapy is difficult to attain, and the effect thereof is usually limited.

Excessive cholesterol in plasma accumulates on the inside of the blood vessels, inducing arteriosclerosis.

However, if the cholesterol is taken up in the liver, and, then cholesterol and its metabolites, namely bile acid are excreted into the intestinal track without accumulation in the liver, followed by discharge together with feces to outside the body, the pool size of cholesterol in the body decreases, to thereby prevent arteriosclerosis onset by the aforementioned mechanism. Thus, a cholesterol-lowering agent which exerts an effect for lowering hepatic cholesterol level as well as plasma cholesterol level has been desired.

A variety of microorganisms exerting a cholesterol-lowering effect with slight side effects are disclosed. However, yeasts are disclosed in surprisingly few number. From long ago, brewer's yeast has been known as a typical yeast, and its effect of improving lipid metabolism is disclosed in several literatures. For example, there are disclosed a case in which administration of chromium-added brewer's yeast (9 g/day) for eight weeks to elderly subjects decreased the serum cholesterol level [Ester G. Offenbacher and F. Xavier Pi-Sunyer, Beneficial effect of chromium-rich yeast on glucose tolerance and blood lipids in elderly subjects. *Diabetes*, 29, 919, (1980)]; a case in which administration of chromium chloride and brewer's yeast (5 g) for 10 weeks to elderly subjects caused no effect on the serum cholesterol level and triacylglycerol level [Caral J. Rinko, and F. Xavier Pi-Sunyer, The effects of inorganic chromium and brewer's yeast on glucose tolerance, plasma lipids, and plasma chromium in elderly subjects., *Am. J. Clin.*

*Nutr.*, 42, 454, (1985)]; and a case in which administration test of no-chromium-added brewer's yeast to human subjects resulted in no effect of lowering the serum cholesterol level [Arne T. Hostmark, Einar Eilertsen, and Ole Gronnerod, Plasma lipid and lipoprotein responses of rats to starch and sucrose diets with and without brewer's yeast., *J. Nutri.*, 109, 1073, (1978)]. In addition, there is disclosed a case in which administration of a soybean-protein-added diet lowered the serum cholesterol level of rats, but a slight increase in the level was attained when 50% of the soybean protein was substituted by brewer's yeast [Jorge De Abreu and Nancy Millan, Effect of addition of brewer's yeast to soy protein and casein on plasma cholesterol levels of rabbits. *Archivos Latinoamericanos de Nutricion.*, 44, 18, (1994)].

As described above, at present, there still remain different theories regarding the effect of brewer's yeast on improvement of lipid metabolism.

Effects of other yeasts are also disclosed in literature. For example, a methanol extract of *Sporobolomyces ruberrinus* and that of *Saccharomyces uvarum* are reported to slightly lower the serum cholesterol level and triglyceride level of cholesterol-loaded rats (Shuji CHO, Hisao FUJII, and Jun Shiraishi, Effect of polysaccharide produced by *Bacillus natto* or alcohol extract of yeast on the lipid metabolism of rats. *Bulletin of the Faculty of Home Life Science, Fukuoka Women's University*, 16, 65, (1984)). Also disclosed is a case in which the culture supernatant of

*Saccharomyces cerevisiae* lowers the serum cholesterol level of mice [Tadaaki Kishida, On the serum Cholesterol-Lowering Effect of *Saccharomyces cerevisiae* Liquid Culture. Jurnal of Japanese Society of Food and Nutrition, 26, 371, (1973)].

However, there is only a few reports in which effect of yeasts other than brewer's yeast or its constituent on improvement of lipid metabolism was demonstrated.

In the human liver, primary bile acids such as cholic acid and chenodeoxycholic acid are synthesized from cholesterol, and conjugated with glycine or taurine, to thereby yield glycine-conjugated or taurine-conjugated bile acid, which are secreted via the bile duct to the digestive tract. Bile acid causes lipid contained in foods and drinks to emulsify and disperse by its surface activation effect, and promotes digestion and absorption of lipid. The bile acid is then actively absorbed again from the ileum, returned to the liver via the portal vein, and resecreted to the digestive tract again. Thus, bile acids repeat enterohepatic circulation.

A portion of bile acid which has not been absorbed by the ileum undergoes modification by enterobacteria; e.g., deconjugation, 7 $\alpha$ -dehydroxylation, oxidation, reduction, or epimerization, to thereby convert to secondary bile acid. Predominant secondary bile acids produced through the process are deoxycholic acid and lithocholic acid, which are formed by 7 $\alpha$ -dehydroxylation of primary bile acids (cholic acid and chenodeoxycholic acid, respectively. A portion of secondary

bile acid is passively absorbed by the large intestine and transferred to the liver, repeating enterohepatic circulation in similar fashion to the case of primary bile acid. The remaining portion of secondary bile acid is excreted into feces.

In recent years, secondary bile acids such as deoxycholic acid and lithocholic acid produced by enterobacteria existing in the large intestinal tract have been found to be closely related to onset of colorectal cancer, liver cancer, pancreatic cancer, bile duct cancer, etc. In general, the carcinogenesis process is conceived to include an initiation step in which a gene is mutated by a chemical carcinogen, radiation, or a virus, and a promotion step in which abnormality occurs in growth and differentiation through long-term exposure to a promoter. Hitherto, there is disclosed that secondary bile acids such as deoxycholic acid and lithocholic acid act as a promoter in the second step, to thereby promote onset of colorectal cancer, liver cancer, pancreatic cancer, bile duct cancer, etc. (Narisawa, T., et al., *J. Natl. Cancer Inst.*, 53, 1093-1097, 1974; Tsuda, H., et al., *Gann*, 75, 871-5, 1984; and Makino, T., et al., *J. Natl. Cancer Inst.*, 76, 967-75, 1986). There is also disclosed that the carcinogenetic promoter activity of secondary bile acid is considerably stronger than that of primary bile acid (Narisawa, T., et al., *J. Natl. Cancer Inst.*, 53, 1093-1097, 1974; and Reddy, BS., et al., *Cancer Res.*, 37, 3238-3242, 1977). Moreover, there has

recently been disclosed that deoxycholic acid may be related to onset of cholelithiasis other than cancers (Marcus, SN., Heaton, KW., *Gut*, 29, 522-533, 1988).

Thus, since secondary bile acids such as deoxycholic acid and lithocholic acid produced by enterobacteria existing in the large intestinal tract have a strong carcinogenetic promoter activity and cause cholelithiasis, diseases such as colorectal cancer, liver cancer, pancreatic cancer, bile duct cancer, and cholelithiasis can be prevented and treated through inhibition of secondary bile acid production.

Hitherto, secondary bile acid production inhibitors have been disclosed, even though the inhibitory effect is weak. These inhibitors are ampicillin—a type of penicillin derivative—(Low-Beer, TS., Nutter, S., *Lancet*, 2(8099), 1063-1065, 1978), 3 $\alpha$ ,12 $\beta$ -dihydroxy-5 $\beta$ -cholane-24-N-methylamine (Roda, A., et al., *J. Pharm. Sci.*, 81, 237-240, 1992); lactulose—a type of oligosaccharide—(Magengast, FM., *Eur. J. Clin. Invest.*, 18, 56-61, 1988); and wheat bran (Lampe, JW., et al., *Gut*, 34, 531-536, 1993).

Ampicillin and 3 $\alpha$ ,12 $\beta$ -dihydroxy-5 $\beta$ -cholane-24-N-methylamine inhibit secondary bile acid production by removing bacteria which catalyze 7 $\alpha$ -dehydroxylation in the large intestinal tract. However, when these inhibitors are employed for a long period of time, there arise problems such as generation of resistant bacteria and occurrence of adverse side effects. In addition, selective exclusion of bacteria which relates to secondary bile acid production by use of

antibiotics is impossible, and use of antibiotics simultaneously excludes enterobacteria which play an important role in maintaining the human health; e.g., *Lactobacillus* and *Bifidobacterium*, and this is greatly problematic.

Lactulose and wheat bran are thought to suppress activity of 7 $\alpha$ -dehydroxylase which has optimal pH of a neutral region by lowering pH in the large intestinal track and thereby lower the production of secondary bile acid. However, these substances exert poor effectiveness, which does not reach the level for contributing to prevention and treatment of the diseases.

In view of the foregoing, an object of the present invention is to provide a cholesterol-lowering agent capable of effectively lowering the blood or hepatic cholesterol level by use of a safe yeast causing fewer and less severe side effects.

Another object of the present invention is to provide a secondary bile acid production inhibitor of high effectiveness and safety, useful for preventing and treating diseases such as colorectal cancer, liver cancer, pancreatic cancer, bile duct cancer, and cholelithiasis.

#### Disclosure of the Invention

The present inventors have carried out extensive studies, and have found that the yeasts belonging to the following specific groups exert an effect of effectively

lowering the blood or hepatic cholesterol and provide fewer and less severe side effects, and that the yeasts exert an effect of inhibiting transformation of primary bile acid to secondary bile acid. The present invention has been accomplished on the basis of these findings.

Accordingly, the present invention provides a cholesterol-lowering agent containing, as an active ingredient, at least one yeast belonging to *Candida*, *Issatchenkia*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, or *Torulaspora*, as well as foods and drinks containing the yeast.

The present invention also provides use of these specific yeasts for producing a cholesterol-lowering agent, as well as foods and drinks for lowering cholesterol.

Further, the present invention provides a treatment method for lowering cholesterol through administration of these specific yeasts.

Still further, the present invention provides a secondary bile acid production inhibitor containing, as an active ingredient, a yeast, as well as foods and drinks containing the yeast.

Yet further, the present invention provides use of the yeast for producing a secondary bile acid production inhibitor, as well as foods and drinks for inhibiting secondary bile acid production.

Still further, the present invention provides a treatment method for inhibiting secondary bile acid

production through administration of a yeast.

#### Brief Description of the Drawing

Fig. 1 is a graph showing plasma cholesterol levels of a cholesterol-added diet administration group and a no-cholesterol-added diet administration group.

#### Best Modes for Carrying out the Invention

The yeast to be used in the cholesterol-lowering agent or foods and drinks according to the present invention is a yeast belonging to *Candida*, *Issatchenkia*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, or *Torulaspora*. These yeast may be used singly or in combination of two or more species. The above yeast may be live cells, heated cells, lyophilized products, milled products thereof, or contents thereof.

Examples of preferred yeasts belonging to the above groups include *Candida kefyr*, *Issatchenkia orientalis*, *Hanseniaspora uvarum*, *Kloeckera africana*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Pichia farinosa*, and *Torulaspora delbrueckii*. Of these, most preferred yeasts are the following:

*Candida kefyr* YIT 8237 (FERM BP-7214, original deposit date July 16, 1999; depository organization, Ministry of International Trade and Industry, National Institute of Bioscience and Human-Technology, Agency of Industrial Science

and Technology (address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566 JAPAN (the same applies hereafter)); *Hanseniaspora uvarum* YIT 8164 (FERM BP-7212, original deposit date July 16, 1999); *Issatchenkia orientalis* YIT 8266 (FERM BP-7215, original deposit date July 16, 1999); *Kloeckera africana* YIT 8165 (FERM BP-7213, original deposit date July 16, 1999), *Kluyveromyces marxianus* YIT 8292 (FERM BP-7217, original deposit date July 16, 1999), *Kluyveromyces lactis* YIT 8263 (FERM BP-7216, original deposit date July 16, 1999), *Pichia farinosa* YIT 8058 (FERM BP-7210, original deposit date July 16, 1999), and *Torulaspora delbrueckii* YIT 8114 (FERM BP-7211, original deposit date July 16, 1999).

From ancient times, these microorganisms have been known to be yeasts for producing foods (wine, milk liquor, cheese), and are strains that are considerably safe to the human body.

The above strains have characteristics as shown in Tables 1 to 5. The properties are nearly the same as those of similar microorganisms disclosed in "The yeast, 3rd edition," (N. J. W. Kreger-Van Rij, Elsevier Science Publishers B. V., Amsterdam, 1984).

Table 1

## Characteristics of Strains

|                                | Strains     | <i>Candida kefyr</i><br>YIT 8237 | <i>Hanseniaspora uvarum</i><br>YIT 8164 | <i>Issatchenka orientalis</i><br>YIT 8266 | <i>Kloeckera africana</i><br>YIT 8165 | <i>Kluyveromyces marxianus</i><br>YIT 8292 | <i>Kluyveromyces lactis</i><br>YIT 8263 | <i>Pichia farinosa</i><br>YIT 8058 | <i>Torulaspor delbrueckii</i><br><sup>a</sup> |
|--------------------------------|-------------|----------------------------------|---|---|---------------------------------------|--|---|------------------------------------|---|
| D-Glucose                      | +           | +                                | +                                       | +   | +                                     | +  | +                                       | +                                  | +   |
| D-Galactose                    | +           | -                                | -                                       | -   | -                                     | +  | -                                       | -                                  | +-  |
| Maltose                        | -           | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | +-  |
| Me- $\alpha$ -D-glucoside      | -           | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | +-  |
| Sucrose                        | +           | -                                | -                                       | -   | -                                     | +-   | -                                       | -                                  | +-  |
| $\alpha$ , $\alpha$ -Trehalose | -           | -                                | -                                       | -   | +                                     | -  | +                                       | -                                  | +-  |
| Fermentation                   | Melibiose   | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | +-  |
| Lactose                        | + -         | -                                | -                                       | -   | -                                     | +-   | -                                       | -                                  | -   |
| Celllobiose                    |             | +                                | -                                       | -   | +-                                    | -  | +-                                      | -                                  | -   |
| Melezitose                     |             | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | -   |
| Raffinose                      | + -         | -                                | -                                       | -   | -                                     | +-   | -                                       | -                                  | +-  |
| Inulin                         | +           | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | +-  |
| Starch                         |             | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | -   |
| D-Xylose                       |             | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | -   |
| D-Glucose                      |             | +                                | +                                       | +   | +                                     | +  | +                                       | +                                  | +   |
| D-Galactose                    | +           | -                                | +-                                      | -   | +                                     | +  | +                                       | +                                  | +   |
| Growth                         | D-Sorbitose | -                                | -                                       | -   | +-                                    | +  | +                                       | +                                  | +-  |
| D-Glucosamine                  |             | -                                | -                                       | -   | -                                     | -  | -                                       | -                                  | +-  |
| D-Ribose                       | + -         | -                                | -                                       | -   | +-                                    | -  | -                                       | +                                  | -   |

Table 2

|                             | Strains | <i>Candida kefyr</i><br>YIT 8237 | <i>Hanseniaspora uvaram</i><br>YIT 8164 | <i>Issatchenkia orientalis</i><br>YIT 8266 | <i>Kloeckera africana</i><br>YIT 8165 | <i>Kluyveromyces marxianus</i><br>YIT 8292 | <i>Kluyveromyces marxianus</i><br>YIT 8263 | <i>Pichia farinosa</i><br>YIT 8058 | <i>Torulaspora delbrueckii</i><br>YIT 8114 |
|-----------------------------|---------|----------------------------------|---|--|---------------------------------------|--|--|------------------------------------|--|
| D-Xylose                    | +-      | -                                | -                                       | -  | +                                     | -  | -  | +-                                 | -  |
| L-Arabinose                 | +-      | -                                | -                                       | -  | -                                     | -  | -  | -                                  | -  |
| D-Arabinose                 | -       | -                                | -                                       | -  | -                                     | -  | -  | -                                  | -  |
| L-Rhamnose                  | -       | -                                | -                                       | -  | +-                                    | -  | +-   | -                                  | -  |
| Sucrose                     | +       | -                                | +                                       | +-   | +                                     | +  | +  | +-                                 | +  |
| Maltose                     | -       | -                                | -                                       | +  | -                                     | +  | -  | +-                                 | +  |
| $\alpha, \alpha$ -Trehalose | +-      | -                                | -                                       | -  | +-                                    | +  | +-   | +                                  | +  |
| Me- $\alpha$ -D-glucoside   | -       | -                                | -                                       | -  | -                                     | +  | -  | +-                                 | +  |
| Celllobiose                 | +-      | +                                | -                                       | +  | +-                                    | +  | +-   | -                                  | -  |
| Salicin                     | +-      | -                                | -                                       | +-   | +                                     | +  | +-   | -                                  | -  |
| Arbutin                     |         | +                                | -                                       | +-   | +                                     | +  | +-   | -                                  | -  |
| Melibiose                   | -       | -                                | -                                       | -  | -                                     | -  | -  | +-                                 | -  |
| Lactose                     | +       | -                                | +                                       | -  | +                                     | +  | +  | -                                  | -  |
| Raffinose                   | +       | -                                | -                                       | -  | -                                     | +  | -  | +-                                 | -  |
| Melezitose                  | -       | -                                | -                                       | -  | -                                     | +  | -  | +-                                 | -  |
| Inulin                      |         | -                                | -                                       | -  | -                                     | -  | -  | +-                                 | -  |
| Starch                      | -       | -                                | -                                       | -  | -                                     | -  | -  | -                                  | -  |
| Glycerol                    | +-      | -                                | +                                       | -  | +                                     | +  | +  | +-                                 | +  |
| Erythritol                  | -       | -                                | -                                       | -  | +                                     | -  | +  | -                                  | -  |
| Ribitol                     | -       | -                                | -                                       | -  | +                                     | -  | +  | -                                  | -  |
| Xylitol                     | -       | -                                | -                                       | -  | +                                     | +  | +  | +-                                 | +  |

Table 3

|                       | Strains | <i>Candida kefyr</i><br>YIT 8237 | <i>Hanseniaspora uvvarum</i><br>YIT 8164 | <i>Issatchenkovia orientalis</i><br>YIT 8266 | <i>Kloeckera africana</i><br>YIT 8165 | <i>Kluyveromyces marxianus</i><br>YIT 8292 | <i>Kluyveromyces lactis</i><br>YIT 8263 | <i>Pichia farinosa</i><br>YIT 8058 | <i>Torulaspora delbrueckii</i><br>YIT 8114 |
|-----------------------|---------|----------------------------------|--|--|---------------------------------------|--|---|------------------------------------|--|
| L-Arabinitol          | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| D-Glucitol            | +-      | +-                               | -  | -  | +                                     | +  | +                                       | -                                  | -  |
| D-Mannitol            | +-      | -                                | -  | -  | +                                     | +  | +                                       | + -                                | + -  |
| Galactitol            | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| myo-Inositol          | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| D-Glucono-1,5-Lactone | +       | -                                | -  | +  | -                                     | -  | +                                       | + -                                | + -  |
| 2-Keto-D-glucconate   | +       | -                                | -  | -  | + -                                   | -  | + -                                     | + -                                | + -  |
| D-Gluconate           | -       | -                                | -  | +  | -                                     | -  | +                                       | -                                  | -  |
| D-Glucronate          | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| D-Galacturonate       | -       | -                                | -  | +  | -                                     | -  | -                                       | -                                  | -  |
| DL-Lactate            | +       | -                                | +  | -  | -                                     | +  | -                                       | -                                  | + -  |
| Succinate             | + -     | -                                | +  | -  | + -                                   | +  | + -                                     | -                                  | -  |
| Citrate               | + -     | -                                | + -                                      | -  | + -                                   | +  | +                                       | -                                  | -  |
| Methanol              | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| Ethanol               | -       | -                                | +  | -  | +                                     | +  | +                                       | +                                  | -  |
| Propane-1,2-diol      | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| Butane-2,3-diol       | -       | -                                | -  | -  | -                                     | +  | -                                       | -                                  | -  |
| Nitrate               | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| Nitrite               | -       | -                                | -  | -  | -                                     | -  | -                                       | + -                                | + -  |
| Ethylamine            | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
| L-Lysine              | -       | -                                | -  | -  | -                                     | -  | -                                       | -                                  | -  |
|                       |         | +                                | +  | +  | +                                     | +  | +                                       | +                                  | +  |

Table 4

|                         | Strains | <i>Candida kefyr</i><br>YIT 8237 | <i>Hanseniaspora uvarum</i><br>YIT 8164 | <i>Issatchenka orientalis</i><br>YIT 8266 | <i>Kloeckera africana</i><br>YIT 8165 | <i>Kluyveromyces marxianus</i><br>YIT 8292 | <i>Kluyveromyces lactis</i><br>YIT 8263 | <i>Pichia farinosa</i><br>YIT 8058 | <i>Torulaspora delbrueckii</i><br>YIT 8114 |
|-------------------------|---------|----------------------------------|---|---|---------------------------------------|--|---|------------------------------------|--|
| Cadaverine              |         | +                                |   |   |                                       | +  | +                                       | +                                  | -  |
| Creatine                |         | -                                | -                                       |   |                                       | -  | -                                       | -                                  | -  |
| Creatinine              |         | -                                | -                                       |   |                                       | -  | -                                       | -                                  | -  |
| Glucosamine             |         | -                                | -                                       |   |                                       | -  | -                                       | -                                  | -  |
| Imidazole               |         | -                                | -                                       |   |                                       | -  | -                                       | -                                  | -  |
| W/O Vitamins            |         | -                                | -                                       |   |                                       | +-   | -                                       | +-                                 | +-   |
| W/O myo-Inositol        |         | -                                | -                                       |   |                                       | +  | +                                       | +                                  | +  |
| W/O Pantothenic acid    |         | -                                | -                                       |   |                                       | +  | +                                       | +                                  | +  |
| W/O Biotin              |         | +-                               | +                                       |   |                                       | +-   | +                                       | +                                  | +-   |
| W/O Thiamine            |         | +-                               | +                                       |   |                                       | +-   | +                                       | +                                  | +  |
| W/O Biotin & Thiamine   |         | -                                | -                                       |   |                                       | +-   | +-                                      | +-                                 | +-   |
| W/O Pyridoxine          |         | -                                | -                                       |   |                                       | +  | +                                       | +                                  | +  |
| W/O Pyridoxine&Thiamine |         | -                                | -                                       |   |                                       | +-   | +-                                      | +-                                 | +-   |
| W/O Niacin              |         | -                                | -                                       |   |                                       | +  | -                                       | +                                  | +  |
| W/O PABA                |         | -                                | -                                       |   |                                       | +  | +                                       | +                                  | +  |
| 25°C                    |         | +                                | +                                       |   |                                       | +  | +                                       | +                                  | +  |
| 30°C                    |         | +                                | +                                       |   |                                       | +  | +                                       | +                                  | +  |
| 35°C                    |         | +                                | -                                       |   |                                       | +  | +                                       | +                                  | +-   |
| 37°C                    |         | +                                | -                                       |   |                                       | -  | +                                       | +                                  | +-   |
| 40°C                    |         | +                                | -                                       |   |                                       | +  | -                                       | +                                  | -  |
| 0.01% Cycloheximide     |         | -                                | -                                       |   |                                       | -  | +                                       | -                                  | -  |

Table 5

|  | Strains                      | <i>Candida kefyr</i><br>YIT 8237 | <i>Hanseniaspora uvarum</i><br>YIT 8164 | <i>Issatchenkia orientalis</i><br>YIT 8266 | <i>Kloeckera africana</i><br>YIT 8165 | <i>Kluyveromyces marxianus</i><br>YIT 8292 | <i>Kluyveromyces lactis</i><br>YIT 8263 | <i>Pichia farinosa</i><br>YIT 8058 | <i>Torulaspora delbrueckii</i><br>YIT 8114 |
|--|------------------------------|----------------------------------|---|--|---------------------------------------|--|---|------------------------------------|--|
| Growth                                       | 0.1% Cycloheximide           | +                                | -                                       | -  | -                                     | -  | +                                       | -                                  | -  |
|  | 1% Acetic acid               | -                                | -                                       | -  | -                                     | -  | -                                       | -                                  | -  |
|  | 50% D-Glucose                | + -                              | -                                       | -  | +                                     | +  | -                                       | +                                  | +  |
|  | 60% D-Glucose                | -                                | -                                       | -  | -                                     | +  | -                                       | +                                  | + -  |
| Addit-<br>ional<br>Chara-<br>cteris-<br>tics | Starch<br>production         | -                                | -                                       | -  | -                                     | -  | -                                       | -                                  | -  |
|  | Acetic acid<br>production    | -                                | -                                       | -  | -                                     | -  | -                                       | -                                  | -  |
|  | Urea<br>hydrolysis           | -                                | -                                       | -  | -                                     | -  | -                                       | -                                  | -  |
|  | Diazonium Blue<br>B reaction | -                                | -                                       | -  | -                                     | -  | -                                       | -                                  | -  |
|  |                              |                                  |   |  |                                       |  |   |                                    |  |

The yeasts which are used in the secondary bile acid production inhibitor of the present invention will next be described.

No particular limitation is imposed on the yeasts which are used in the secondary bile acid production inhibitor.

The yeasts include live cells produced by culturing the yeasts, heated cells, lyophilized products, milled products thereof, and contents thereof.

The yeast to be used in the present invention has never been reported to inhibit elimination of the  $7\alpha$ -hydroxyl group of cholic acid or similar substances, to thereby inhibit formation of secondary bile acid such as deoxycholic acid. Since the present invention employs the effect of yeast on suppression and inhibition of transformation of primary bile acid to secondary bile acid, yeasts which have excellent absorption power to primary bile acids, such as, particularly, cholic acid, taurocholic acid, glycocholic acid, and chenodeoxycholic acid, as well as excellent power to elevate the intestinal concentration level of short-chain fatty acids such as acetic acid, propionic acid, and butyric acid are preferred, from the viewpoint of excellent effect of inhibiting  $7\alpha$ -dehydroxylation.

Examples of yeasts preferably used in the secondary bile acid production inhibitor include *Issatchenkia*, *Kluyveromyces*, *Hanseniaspora*, *Saccharomyces*, *Hyphopichia*, *Candida*, *Torulaspora*, *Pichia*, and *Zygosaccharomyces*. These yeasts may be used singly or in combination of two or more

species.

Specific examples include *Issatchenkia orientalis*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces thermotolerans*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Saccharomyces dairensis*, *Saccharomyces exiguum*, *Saccharomyces unisporus*, *Saccharomyces bayanus*, *Hyphopichia burtonii*, *Candida kefyr*, *Candida etchellsii*, *Candida zeylanoides*, *Candida solani*, *Candida maltosa*, *Candida tropicalis*, *Candida cylindracea*, *Candida utilis*, *Torulaspora delbrueckii*, *Pichia anomala*, *Pichia holstii*, and *Zygosaccharomyces rouxii*. Of these, most preferred yeasts are the following:

*Issatchenkia orientalis* YIT 8266 (FERM BP-7215);  
*Kluyveromyces marxianus* YIT 8292 (FERM BP-7217);  
*Kluyveromyces lactis* YIT 8263 (FERM BP-7216); *Kluyveromyces thermotolerans* YIT 8294 (ATCC 20309); *Hanseniaspora uvarum* YIT 8164 (FERM BP-7212); *Saccharomyces cerevisiae* YIT 8116 (ATCC 48554); *Saccharomyces dairensis* YIT 8191 (CBS 3007);  
*Saccharomyces exiguum* YIT 8109 (CBS 3019); *Saccharomyces unisporus* YIT 8226 (FERM BP-7209, original deposit date; March 25, 1999, depository organization, Ministry of International Trade and Industry, National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566 JAPAN); *Saccharomyces bayanus* YIT 8128 (CBS 380); *Hyphopichia burtonii* YIT 8299 (CBS 2352); *Candida kefyr* YIT 8237 (FERM BP-7214); *Candida etchellsii* YIT 8278

(ATCC 60119); *Candida zeylanoides* YIT 8018 (IFO 0719); *Candida solani* YIT 8023 (CBS 1908); *Candida maltosa* YIT 8283 (ATCC 28140); *Candida tropicalis* YIT 8286 (CBS 94); *Candida cylindracea* YIT 8276 (ATCC 14830); *Candida utilis* YIT 8204 (ATCC 9950); *Torulaspora delbrueckii* YIT 8313 (JCM 2204) and YIT 8133 (IFO 1172); *Pichia anomala* YIT 8298 (JCM 3587) and YIT 8297 (JCM 3583); *Pichia holstii* YIT 8038 (ATCC 58048); and *Zygosaccharomyces rouxii* YIT 8129 (CBS 732).

From ancient times, these yeasts have been used to produce foods (wine, cheese), and are considerably safe microorganisms to the human body. The cells thereof, which are particles having a particle size of some microns, are much easily taken as compared with particles having a size of 20  $\mu\text{m}$  or more, which provide unfavorable sensation when taken. The above yeasts; e.g., *Issatchenka orientalis* YIT 8266 (FERM BP-7215), have specific characteristics as shown in Table 6. The characteristics are nearly the same as those of similar yeasts disclosed in "The yeast, 3rd edition," (N. J. W. Kreger-Van Rij, Elsevier Science Publishers B. V., Amsterdam, 1984).

Table 6

Characteristics of *Issatchenkovia orientalis* YIT 8266

| Fermentation                | Melibiose | -                     | Creatine | -                          |
|-----------------------------|-----------|-----------------------|----------|----------------------------|
| D-Glucose                   | +         | Lactose               | +        | Creatinine                 |
| D-Galactose                 | -         | Raffinose             | -        | Glucosamine                |
| Maltose                     | -         | Melezitose            | -        | Imidazole                  |
| Me- $\alpha$ -D-glucose     | -         | Inulin                | -        | W/O Vitamin                |
| Sucrose                     | -         | Starch                | -        | W/O <i>myo</i> -Inositol   |
| $\alpha, \alpha$ -Trehalose | -         | Glycerol              | +        | W/O Pantothenic acid       |
| Melibiose                   | -         | Erythritol            | -        | W/O Biotin                 |
| Lactose                     | -         | Ribitol               | -        | W/O Thiamin                |
| Cellobiose                  | -         | Xylitol               | -        | W/O Biotin & thiamin       |
| Melezitose                  | -         | L-Arabinitol          | -        | W/O Pyridoxine             |
| Raffinose                   | -         | D-Glucitol            | -        | W/O Pyridoxine & thiamin   |
| Inulin                      | -         | D-Mannitol            | -        | W/O Niacin                 |
| Starch                      | -         | Galactitol            | -        | W/O PABA                   |
| D-Xylose                    | -         | <i>myo</i> -Inositol  | -        | 25°C                       |
| Growth                      |           |                       |          |                            |
| D-Glucose                   | +         | D-Glucono-1,5-lactone | -        | 30°C                       |
| D-Galactose                 | ++        | 2-Keto-D-gluconate    | -        | 35°C                       |
| L-Sorbose                   | -         | D-Gluconate           | -        | 37°C                       |
| D-Glucosamine               | -         | D-Glucuronate         | -        | 40°C                       |
| D-Ribose                    | -         | D-Galacturonate       | -        | 0.01%                      |
| D-Xylose                    | -         | DL-Lactate            | +        | Cycloheximide              |
| L-Arabinose                 | -         | Succinate             | +        | 0.1% Cycloheximide         |
| D-Arabinose                 | -         | Citrate               | +        | 1% Acetic acid             |
| L-Rhamnose                  | -         | Methanol              | -        | 50% D-Glucose              |
| Sucrose                     | -         | Ethanol               | +        | 60% D-Glucose              |
| Maltose                     | -         | Propane-1,2-diol      | -        | Additional characteristics |
| $\alpha, \alpha$ -Trehalose | -         | Butane-2,3-diol       | -        | Starch production          |
| Me- $\alpha$ -D-glucoside   | -         | Nitrate               | -        | Acetic acid production     |
| Cellobiose                  | -         | Nitrite               | -        | Urea hydrolysis            |
| Salcin                      | -         | Ethylamine            | +        | Diazonium Blue B reaction  |
| Arbutin                     | -         | L-Lysine              | +        |                            |
|                             | -         | Cadaverine            | +        |                            |

The yeast of the present invention can be produced through a routine method; e.g., culturing in a complex medium containing yeast extract and polypeptone or in a synthetic

medium predominantly containing an inorganic salt.

The thus-produced yeast can be employed, in the forms of live cells, lyophilized products thereof, killed cells obtained through heating or similar treatment, milled products thereof, or contents thereof, in a pharmaceutical composition or foods and drinks. In addition, commercially available yeast may also be used.

The aforementioned yeast may be formed into a pharmaceutical composition of a variety of drug foams, through a routine method together with pharmaceutically acceptable carriers. When a solid drug for peroral administration is prepared, the aforementioned yeast is mixed with a vehicle and optional additives such as a binder, a disintegrant, a lubricant, a colorant, a flavoring agent, and an aroma, and the resultant mixture is formed, through a customary method, into tablets, coated tablets, granules, powder, or capsules. Additives generally employed in the art may be used as the above additives. Examples include vehicles such as lactose, white sugar, sodium chloride, glucose, starch, calcium carbonate, kaolin, microcrystalline cellulose, and silicic acid; binders such as water, ethanol, propanol, simple syrup, glucose solution, starch solution, gelatin solution, carboxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl starch, methyl cellulose, ethyl cellulose, calcium phosphate, and poly(vinylpyrrolidone); disintegrants such as dry starch, sodium alginate, powdered agar, sodium hydrogencarbonate, calcium carbonate, sodium

lauryl sulfate, stearic monoglyceride, and lactose; lubricants such as purified talc, stearate salts, borax, and polyethylene glycol; and flavoring agents such as white sugar, orange peel, citric acid, and tartaric acid.

When a liquid drug for peroral administration is prepared, the aforementioned yeast is mixed with additives such as a flavoring agent, a buffer, a stabilizer, and an aroma, and the resultant mixture is formed, through a customary method, into peroral liquid, syrups, and elixirs. The aforementioned flavoring agents may also be used.

Examples of the buffer include sodium citrate, and examples of the stabilizer include tragacanth, gum arabic, and gelatin.

The foods and drinks of the present invention can be produced by adding the aforementioned yeast to a variety of foods and drinks. Examples of preferred foods and drinks include fermented milk, fruit-juice-blended drinks, soup, rice cakes, and cookies. Animals feeds are also included in the foods and drinks.

The amount of yeast which is to be added to the aforementioned drug varies in accordance with the drug form and the condition of the patient to which the drug is to be administered. Generally, an amount of 1-100 wt.% in the drug is preferred. The daily dose of the aforementioned drug or foods and drinks varies in accordance with the condition, body weight, age, sex, etc. of the patient, and cannot be determined as a fixed value. Generally, the daily dose per adult is approximately 10 mg to 30 g based on yeast,

preferably approximately 1-5 g. The yeast is administered preferably once per day or 2-4 times per day in a divided manner.

## EXAMPLES

The present invention will next be described in more detail by way of examples, which should not be construed as limiting the invention thereto. As used herein, "%" refers to "wt.%".

### Example 1

#### (A) Preparation of yeasts

Each of a variety of yeasts, which had been stored in a potato dextrose agar slant medium, was inoculated for one platinum loop into a medium (100 mL) shown in Table 7 placed in a Sakaguchi flask (500 mL), and the medium was subjected to shaking culture (120 spm) at 30°C.

Two days after initiation of culture, the culture (amount corresponding to two Sakaguchi flasks) was inoculated into a 10-liter fermentation tank (effective volume 7 L), and aerobic agitation culture was carried out at 30°C for two days, under the following conditions: aeration rate of 0.5 vvm, rotation rate of 250 rpm, and pH of 6.0 (automatically controlled by 5N sodium hydroxide).

After completion of culturing, the supernatant and cells were separated by means of a cooling centrifugator, and the separated cells were washed twice by distilled water. The washed cells were placed in a 2-L Erlenmeyer flask, and

distilled water (1 L) was added thereto. The mixture was heated at 115°C for 10 minutes in an autoclave. The thus-heated cells as such were lyophilized.

Table 7  
Composition of Medium for Pre-Culture and Culture

|  |       |
|--|-------|
| Glucose (Kanto Chemical)                     | 30 g  |
| Polypeptone (Daigo Eiyo)                     | 10 g  |
| Yeast extract (Daigo Eiyo)                   | 5 g   |
| Monopotassium phosphate (Wako Pure Chemical) | 1 g   |
| Dipotassium phosphate (Wako Pure Chemical)   | 2 g   |
| Magnesium sulfate (Wako Pure Chemical)       | 0.5 g |
| Tap water                                    | 1 L   |
| pH 6.0                                       |       |

(B) Preparation of test diets

By use of the yeast produced in (A), ingredients were mixed in a routine manner, to thereby prepare diets having a composition shown in Table 8. Each diet contains cholesterol; i.e., cholesterol (0.5%) and sodium cholate (0.25%). In order to prepare a yeast-containing diet, each type of lyophilized yeast cells was added in an amount of 5% or 10 %.

Table 8

| Ingredients         | Diet Composition (%) |                                |                               |
|---------------------|----------------------|--------------------------------|-------------------------------|
|                     | Control group        | 10% Yeast administration group | 5% Yeast administration group |
| Casein              | 22.30                | 17.35                          | 19.83                         |
| Soybean oil         | 1.0                  | 1.0                            | 1.0                           |
| Lard                | 10.0                 | 9.45                           | 9.72                          |
| Inorganic salt      | 4.0                  | 3.54                           | 3.77                          |
| Vitamins            | 1.0                  | 1.0                            | 1.0                           |
| Choline bitartrate  | 0.15                 | 0.15                           | 0.15                          |
| Cholesterol         | 0.5                  | 0.5                            | 0.5                           |
| Sodium cholate      | 0.25                 | 0.25                           | 0.25                          |
| Filter paper powder | 10.0                 | 7.46                           | 8.73                          |
| Sucrose             | 50.80                | 50.12                          | 50.46                         |
| Yeast cells         | 0.0                  | 10.0                           | 5.0                           |

## Test method      Cholesterol-lowering effect

## (1) Test diet

Test diets prepared in Example 1 (B) were employed.

## (2) Animal and breeding method

a) Group of animals administered 10% yeast-containing diet

Male, 5-week-old Wistar rats (obtained from Clea Japan) had been pre-bred for 7 days with diet powder F-2 (product of Oriental Yeast). The rats were divided into groups, each group containing 8 rats. To each group, the 10% yeast-containing diet shown in Table 8 was administered for 7 days. The rats were bred in group in a metal-made cage, and allowed to consume the administered diet and water *ad libitum*.

b) Group of animals administered 5% yeast-containing diet

Male, 5-week-old Wistar rats (obtained from Nihon Kurea) had been pre-bred for 7 days with diet powder F-2 (product of Oriental Yeast). The rats were divided into groups, each group containing 8 rats. To each group, the 5% yeast-containing diet shown in Table 8 was administered for 14 days. The rats were bred individually in a metal-made cage, and the amount of administered diet was limited to 15 g/day, but water was provided *ad libitum*.

(3) Measurement of plasma lipids

a) Group of animals administered 10% yeast-containing diet

Seven days after initiation of administration, blood was collected, under anesthetization with Nembutal (without fasting), from the abdominal aorta of each rat by use of a cannula, and the plasma cholesterol concentration and the plasma HDL concentration were measured. The total cholesterol concentration was measured by means of a biochemical automatic analyzer (Model 7170, Hitachi).

The HDL cholesterol concentration was measured by means of a biochemical automatic analyzer (Model 7170, Hitachi), after lipoprotein components other than HDL had been precipitated by use of Determiner HDL (product of Kyowa Medics).

b) Group of animals administered 5% yeast-containing diet

Seven days after initiation of administration, blood was collected from the tail vein of each rat, and the plasma

cholesterol concentration and the plasma HDL concentration were measured. The total cholesterol concentration was measured by means of a biochemical automatic analyzer (Model 7170, Hitachi).

The HDL cholesterol concentration was measured by means of a biochemical automatic analyzer (Model 7170, Hitachi), after lipoprotein components other than HDL had been precipitated by use of Determiner HDL (product of Kyowa Medics).

#### (4) Measurement of intrahepatic lipids

After perfusion of the liver by use of physiological saline, the liver was collected and lyophilized. Lipid extraction was performed by use of chloroform : methanol (2 : 1) in accordance with the method of Folch *et al.* The resultant chloroform lower layer was concentrated to solid, and the solid was diluted again by use of ethanol. The diluted sample was subjected to measurement of lipid components contained in the liver. Among hepatic lipids, the amount of cholesterol was measured by use of Determiner TC 555.

Method for evaluating animal test results

#### (1) Statistical method

Upon statistical analysis of the animal test results, homogeneity of variance was tested by the Bartlett method. When the variance was found to be homoscedastic, analysis of variance was performed in a one-way layout manner, followed by Dunnett's multiple comparison test, whereas when the

variance was found to be heteroscedastic (test objects from which significant difference was obtained through Bartlett test at a level of significance under 5 %), analysis of variance was performed through the Kruskal-Wallis test, followed by Dunnett's multiple comparison test. In Dunnett's multiple comparison test, the level of significance was set at 5% and 1%, and the test was performed at each level of significance.

(2) Method for obtaining percent reduction of lipid components

<Percent reduction in plasma cholesterol>

The expression "100% reduction in plasma cholesterol" refers to a plasma cholesterol level lowered to a plasma cholesterol level of a rat belonging to a group administered an ordinary diet (no-cholesterol-added). The express "0% reduction in plasma cholesterol" refers to a plasma cholesterol level elevated to a plasma cholesterol level of a rat belonging to a group administered a cholesterol-added diet (control).

(3) Atherogenic index

Atherogenic index (AI) is represented by the following formula:

AI = (VLDL cholesterol + LDL cholesterol)/HDL cholesterol.  
AI was calculated from the total plasma cholesterol level and the plasma HDL cholesterol; i.e., actual plasma lipid measurements, according to the following formula:

$$AI = ((\text{total plasma cholesterol}) - (\text{plasma HDL})) / (\text{plasma HDL})$$

cholesterol))/(plasma HDL cholesterol).

The percent improvement of atherogenic index was calculated from the AI value of each yeast-containing-diet administration group and the AI value of cholesterol-added-diet administration group (control group). The "expression 100% improvement of atherosclerotic index" refers to an AI value identical to the AI value of the no-cholesterol-added-diet administration group. The expression "0% improvement of atherosclerotic index" refers to an AI value identical to the AI value of the cholesterol-added-diet administration group (control group).

#### (4) Body weight measurement

The body weight of each rat was measured upon purchase thereof. The rats were separated into groups such that no significant difference in body weight was present among the groups, and pre-breeding was started. The body weight was measured before starting the test and after completion of the test.

The aforementioned test method was adopted to the following pre-tests and Examples.

#### Preliminary test:

Blood was collected from the tail vein of rats after administration of the cholesterol-added diet; i.e., on day 0, day 5, and day 8. On day 14, blood was collected, under anesthetization with Nembutal, from the abdominal aorta. Plasma was separated from each collected blood sample, and subjected to lipid component measurement through an enzyme

method.

As shown in Fig. 1, the plasma cholesterol level of the cholesterol-added diet administration group increased to approximately the maximum at day 5. Thereafter, a slight increase was observed until day 14, but no significant change was observed. The plasma cholesterol level of the no-cholesterol-added diet administration group (diet prepared by removing cholesterol and sodium cholate from the cholesterol-added diet) did not increase during the test period, and a tendency of slight decrease in level was observed.

Table 9 shows the plasma lipid concentration on day 14 after initiation of administration of cholesterol-added diet. The cholesterol level of the no-cholesterol-added diet administration group is 72.5 mg/dL, whereas that of the cholesterol-added diet administration group is 325.3 mg/dL, indicating an approximately 4.5 fold increase in level. In contrast, the plasma HDL cholesterol level of the cholesterol-added diet administration group is 45.9 mg/dL, significantly lower than that of the no-cholesterol-added diet administration group (31.4 mg/dL).

Table 9

| Plasma Lipid Concentration (day 14)                            |                     |                         |
|--|---------------------|-------------------------|
| Diet administration groups                                     | Cholesterol (mg/dL) | HDL cholesterol (mg/dL) |
| Cholesterol free diet<br>(Control group)                       | 72.5 ± 6.1          | 45.9 ± 4.2              |
| Cholesterol added diet<br>(high-fat diet administration group) | 325.3 ± 48.6**      | 31.4 ± 4.00**           |

\*\*p<0.01

Table 10 shows the hepatic lipid content on day 14 after initiation of administration of the cholesterol-added diet. The cholesterol level of the cholesterol-added diet administration group is about 20 times the cholesterol level of the no-cholesterol-added diet administration group.

Table 10

| Intrahepatic Lipid Content (day 14)                            |                              |
|--|------------------------------|
| Diet administration groups                                     | Total cholesterol (mg/liver) |
| Cholesterol free diet<br>(Control group)                       | 32.9 ± 8.4                   |
| Cholesterol added diet<br>(high-fat diet administration group) | 637.9 ± 60.70**              |

\*\*p<0.01

#### Example 2

Jar culture was performed in accordance with the method of Example 1, to thereby prepare 200 g of lyophilized cells (heated at 110°C for 10 minutes) of each yeast. Each cell sample was mixed into a cholesterol-added diet at 10% (10% yeast group in Table 8). The resultant diet was administered to each group consisting of eight rats, and consumed ad

*libitum* for seven days.

Tables 11 to 13 show the percent reduction of cholesterol level and the percent improvement (%) of atherosclerotic index, based on the plasma cholesterol level of the high-cholesterol-diet administration control group. No significant change in weight was observed among the tested groups.

Table 11

Percent Reduction of Plasma Cholesterol and Percent Improvement of AI

| YIT No. | Yeast taxonomic genus and species | Percent Reduction of Plasma Cholesterol and Percent Improvement of AI |                     |                               | Av. body weight (g) |
|---------|-----------------------------------|---|---------------------|-------------------------------|---------------------|
|         |                                   | Percent reduction of cholesterol                                      | Dunnett's test (p<) | Percent improvement of AI (%) |                     |
| 8164    | <i>Hanseniaspora uvarum</i>       | 76.5  | 0.01                | 78.0                          | 224.0               |
| 8301    | <i>Pichia guilliermondii</i>      | 74.7  | 0.01                | 60.1                          | 216.4               |
| 8263    | <i>Kluyveromyces lactis</i>       | 72.8  | 0.01                | 68.8                          | 220.4               |
| 8292    | <i>Kluyveromyces marxianus</i>    | 65.5  | 0.01                | 55.8                          | 219.6               |
| 8266    | <i>Issatchenkia orientalis</i>    | 64.2  | 0.01                | 55.3                          | 225.0               |
| 8299    | <i>Hyphopichia burtonii</i>       | 62.7  | 0.01                | 54.0                          | 219.0               |
| 8058    | <i>Pichia farinosa</i>            | 60.3  | 0.05                | 51.8                          | 212.1               |
| 8237    | <i>Candida kefyr</i>              | 60.0  | 0.01                | 59.3                          | 216.4               |
| 8165    | <i>Kloeckera africana</i>         | 54.9  | n.s.                | 54.1                          | 216.8               |
| 8313    | <i>Torulaspora delbrueckii</i>    | 52.7  | 0.01                | 51.0                          | 221.4               |
| 8114    | <i>Torulaspora delbrueckii</i>    | 52.5  | 0.05                | 46.2                          | 217.4               |
| 8298    | <i>Pichia anomala</i>             | 50.7  | 0.05                | 38.0                          | 223.2               |
| 8280    | <i>Candida glabrata</i>           | 48.1  | 0.05                | 42.9                          | 215.0               |
| 8104    | <i>Kluyveromyces marxianus</i>    | 48.0  | 0.05                | 44.9                          | 221.0               |
| 8278    | <i>Candida etchellensis</i>       | 46.1  | 0.01                | 41.5                          | 224.2               |
| 8026    | <i>Debaryomyces hansenii</i>      | 44.5  | 0.05                | 30.8                          | 217.0               |
| 8018    | <i>Candida zeylanoides</i>        | 43.5  | 0.05                | 48.4                          | 218.4               |
| 8007    | <i>Candida krusei</i>             | 38.3  | 0.05                | 43.8                          | 225.6               |
| 8223    | <i>Candida gropengiesseri</i>     | 38.1  | n.s.                | 37.2                          | 220.0               |

Table 12 Percent Reduction of Plasma Cholesterol and Percent Improvement of AI

| YIT No. | Yeast taxonomic genus and species   | Percent reduction of cholesterol |                |                               | Plasma lipid                |                               | Av. body weight (g) |
|---------|-------------------------------------|----------------------------------|----------------|-------------------------------|-----------------------------|-------------------------------|---------------------|
|         |                                     | Percent reduction of cholesterol | Dunnett's test | Percent improvement of AI (%) | Percent reduction of AI (%) | Percent improvement of AI (%) |                     |
| 8247    | <i>Pichia capsulata</i>             | 38.1                             | n.s.           | 22.6                          |                             |                               | 220.5               |
| 8294    | <i>Kluyveromyces thermotolerans</i> | 37.7                             | n.s.           | 63.4                          |                             |                               | 220.4               |
| 8159    | <i>Candida magnoliae</i>            | 37.1                             | n.s.           | 51.8                          |                             |                               | 220.6               |
| 8281    | <i>Candida gropengiesseri</i>       | 36.5                             | 0.05           | 34.6                          |                             |                               | 224.0               |
| 8006    | <i>Candida parapsilosis</i>         | 36.4                             | n.s.           | 33.8                          |                             |                               | 218.0               |
| 8314    | <i>Yarrowia lipolytica</i>          | 35.8                             | n.s.           | 24.5                          |                             |                               | 208.2               |
| 8110    | <i>Torulaspora delbrueckii</i>      | 34.5                             | n.s.           | 23.5                          |                             |                               | 217.0               |
| 8133    | <i>Torulaspora delbrueckii</i>      | 33.7                             | n.s.           | 32.3                          |                             |                               | 209.8               |
| 8300    | <i>Pichia etchellsii</i>            | 33.3                             | n.s.           | 52.0                          |                             |                               | 223.2               |
| 8302    | <i>Pichia membranaefaciens</i>      | 31.5                             | n.s.           | 46.6                          |                             |                               | 220.4               |
| 8241    | <i>Candida valida</i>               | 30.4                             | n.s.           | 34.0                          |                             |                               | 223.2               |
| 8303    | <i>Pichia subpelluculosa</i>        | 27.7                             | n.s.           | 48.2                          |                             |                               | 221.4               |
| 8285    | <i>Candida stellata</i>             | 26.8                             | n.s.           | 21.3                          |                             |                               | 219.0               |
| 8277    | <i>Candida diversa</i>              | 26.0                             | n.s.           | 28.6                          |                             |                               | 226.4               |
| 8291    | <i>Issatchenkia terricola</i>       | 25.2                             | n.s.           | 30.1                          |                             |                               | 212.2               |
| 8107    | <i>Torulaspora delbrueckii</i>      | 24.8                             | n.s.           | 20.2                          |                             |                               | 207.8               |
| 8034    | <i>Williopsis californica</i>       | 23.2                             | n.s.           | 10.2                          |                             |                               | 214.8               |
| 8023    | <i>Candida solani</i>               | 22.9                             | n.s.           | 25.0                          |                             |                               | 226.6               |
| 8288    | <i>Candida vini</i>                 | 21.8                             | n.s.           | 20.7                          |                             |                               | 223.8               |
| 8033    | <i>Pichia anomala</i>               | 20.4                             | n.s.           | 23.0                          |                             |                               | 220.6               |

Table 13

Percent Reduction of Plasma Cholesterol and Percent Improvement of AI

| YIT No. | Yeast taxonomic genus and species     | Percent Reduction of Plasma Cholesterol |                     |                               | Percent Improvement of AI |                     |                               |
|---------|---------------------------------------|---|---------------------|-------------------------------|---------------------------|---------------------|-------------------------------|
|         |                                       | Percent reduction of cholesterol (%)    | Dunnett's test (p<) | Percent improvement of AI (%) | Plasma lipid              | Dunnett's test (p<) | Percent improvement of AI (%) |
| 8012    | <i>Yarrowia lipolytica</i>            | 13.3                                    | n.s.                | 22.4                          |                           |                     | 216.6                         |
| 8181    | <i>Rhodotorula minuta</i>             | 12.2                                    | n.s.                | -15.4                         |                           |                     | 224.0                         |
| 8304    | <i>Rhodotorula glutinis</i>           | 1.0                                     | n.s.                | -13.4                         |                           |                     | 221.0                         |
| 8312    | <i>Saccharomyces malanga</i>          | -2.1                                    | n.s.                | -11.1                         |                           |                     | 215.6                         |
| 8120    | <i>Pachytichospora transvaalensis</i> | -9.7                                    | n.s.                | -24.3                         |                           |                     | 207.8                         |
| 8317    | <i>Zygosaccharomyces rouxii</i>       | -10.2                                   | n.s.                | 0.6                           |                           |                     | 216.8                         |
| 8129    | <i>Zygosaccharomyces rouxii</i>       | -34.2                                   | n.s.                | -55.9                         |                           |                     | 199.2                         |
| 8315    | <i>Zygosaccharomyces bailii</i>       | -67.5                                   | n.s.                | -77.9                         |                           |                     | 209.0                         |

Example 3: 10% Yeast-added diet administration test

Test method;

Jar culture was performed in accordance with the method of Example 1 (A), to thereby prepare 200 g of lyophilized cells of each yeast. Each cell sample was mixed into a cholesterol-added diet at 10% (10% yeast group in Table 8). The resultant diet was administered to each group consisting of eight rats, and consumed *ad libitum* for seven days. Table 14 shows the results of plasma lipid measurement.

Table 14

Plasma lipid (10% mixed administration)

| Strains  | Total cholesterol (mg/dL) | HDL cholesterol (mg/dL) | Reduction of cholesterol (%) | AI (-)        | Percent improvement of AI (%) |
|--|---------------------------|-------------------------|------------------------------|---------------|-------------------------------|
| Control group (high-fat-diet administration group) | 226.0 ± 14.9              | 21.7 ± 3.4              | —                            | 9.62 ± 1.75   | —                             |
| <i>Kluyveromyces marxianus</i> YIT 8292            | 134.5 ± 29.7**            | 23.2 ± 3.9              | 65.5                         | 4.84 ± 1.22** | 55.8                          |
| <i>Issatchenka orientalis</i> YIT 8266             | 136.2 ± 18.7**            | 23.4 ± 3.0              | 64.2                         | 4.88 ± 0.99** | 55.3                          |
| Control group (high-fat diet administration group) | 244.5 ± 50.3              | 23.7 ± 3.4              | —                            | 9.43 ± 2.33   | —                             |
| <i>Kluyveromyces lactis</i> YIT 8263               | 126.8 ± 26.4**            | 27.2 ± 1.8              | 72.8                         | 3.67 ± 1.06** | 68.8                          |
| Control group (high-fat diet administration group) | 230.1 ± 26.9              | 25.9 ± 3.5              | —                            | 7.97 ± 1.17   | —                             |
| <i>Hanseniaspora uvvarum</i> YIT 8164              | 127.5 ± 19.4**            | 35.9 ± 4.6**            | 76.5                         | 2.58 ± 0.54** | 78.0                          |
| <i>Candida kefyr</i> YIT 8237                      | 133.8 ± 19.4**            | 27.8 ± 2.4              | 60.0                         | 3.87 ± 1.06** | 59.3                          |
| Control group (high-fat diet administration group) | 222.0 ± 14.9              | 23.5 ± 6.1              | —                            | 8.94 ± 3.10   | —                             |
| <i>Torulaspora delbrueckii</i> YIT 8114            | 148.1 ± 19.9**            | 23.7 ± 1.9              | 52.5                         | 5.30 ± 1.05** | 46.2                          |
| <i>Pichia farinosa</i> YIT 8054                    | 140.6 ± 18.7**            | 24.8 ± 4.8              | 60.3                         | 4.86 ± 2.07** | 51.8                          |
| <i>Kloeckera africana</i> YIT 8165                 | 143.7 ± 37.5**            | 25.4 ± 1.9              | 54.9                         | 4.68 ± 1.26** | 54.1                          |

\*\*p&lt;0.01

Through administration of a cholesterol-added high-fat food, the control group exhibited increase in total cholesterol level and elevation of atherosclerotic index to 7.97-9.62. However, the test groups—rats to which a diet containing the yeast cells according to the present invention (10%) had been administered—were found to remarkably suppress elevation of the values in terms of all analyzed items, even though the same high-fat diet was administered. Thus, yeasts; i.e., *Kluyveromyces marxianus* YIT 8292, *Kluyveromyces lactis* YIT 8263, *Hanseniaspora uvarum* YIT 8164, *Issatchenka orientalis* YIT 8266, *Candida kefyr* YIT 8237, and *Pichia farinosa* YIT 8058, exhibited remarkably excellent percent reduction of plasma cholesterol and were found to improve atherogenic index.

Example 4: 5% Yeast-added diet administration test

The procedure of Example 3 employing male 5-week-old Wistar rats was repeated, except that each lyophilized cell sample was mixed into a diet for test groups at 5% (5% yeast group in Table 8); the resultant diet was administered to each group consisting of eight rats; with restricted amount (15 g/day) for 14 days.

Table 15 shows the results of plasma lipid analysis. As a result of administration of a high-fat food, the control group exhibited increase in total cholesterol level and elevation of atherogenic index. However, the test groups—rats to which a diet containing the yeast cells according to the present invention (5%) had been

administered—were found to remarkably suppress elevation of the values in terms of all analyzed items, even though the same high-fat diet was administered.

Table 15

| Strains   | Plasma lipid   |  |                             | Percent improvement of AI (%)  |
|---|--|--|-----------------------------|--|
|   | Total cholesterol (mg/dL)  | HDL cholesterol (mg/dL)                              | Percent reduction of AI (-) |  |
| Control group (high-fat diet administration group)<br><i>Kluyveromyces marxianus</i> YIT 8292   | 339.7 ± 34.8<br>205.3 ± 30.1**                                     | 23.2 ± 3.8<br>23.6 ± 5.2                             | —<br>57.6                   | 13.85 ± 1.91<br>7.87 ± 1.18**<br>—<br>46.8   |
| Control group (high-fat diet administration group)<br><i>Kluyveromyces lactis</i> YIT 8263<br><i>Candida kefyr</i> YIT 8237<br><i>Kloeckera africana</i> YIT 8165 | 377.8 ± 75.3<br>222.5 ± 27.2**<br>228.9 ± 69.1**<br>276.9 ± 54.6** | 21.9 ± 1.9<br>23.9 ± 4.4<br>25.4 ± 5.0<br>25.9 ± 5.2 | —<br>55.3<br>53.0<br>36.1   | 16.33 ± 3.83<br>8.59 ± 2.01**<br>7.98 ± 1.74**<br>9.89 ± 2.30**<br>—<br>50.7<br>54.7<br>42.1 |
| Control group (high-fat diet administration group)<br><i>Hanseniaspora uvvarum</i> YIT 8164<br><i>Issatchenkia orientalis</i> YIT 8266                            | 360.0 ± 44.7<br>237.8 ± 34.8**<br>241.5 ± 49.6**<br>243.3 ± 38.5** | 21.5 ± 5.2<br>24.2 ± 2.8<br>20.9 ± 2.8<br>24.5 ± 3.1 | —<br>46.5<br>45.1<br>44.3   | 18.37 ± 6.81<br>8.45 ± 2.45**<br>9.95 ± 1.56**<br>8.86 ± 2.09**<br>—<br>57.3<br>48.7<br>55.0 |
| Control group (high-fat diet administration group)<br><i>Torulaspora delbrueckii</i> YIT 8114   | 284.2 ± 54.1<br>199.2 ± 37.3**                                     | 19.9 ± 4.5<br>21.9 ± 5.3                             | —<br>45.4                   | 13.66 ± 3.34<br>8.63 ± 3.41**<br>—<br>40.0   |

\*\*p&lt;0.01

Table 16 shows the results of hepatic lipid measurement. In all test groups, the total hepatic cholesterol level decreased significantly, as compared with the control group. Thus, the yeasts according to the present invention have been found to serve as an excellent anti-cholesterol material which exerts an effect of lowering plasma lipid through peroral administration, as well as an effect of lowering hepatic lipid.

Table 16

| Strains   | Total cholesterol<br>(mg/liver) |
|---|---------------------------------|
| Control group<br>(high-fat diet administration group) | 538.9 ± 53.5                    |
| <i>Kluyveromyces marxianus</i> YIT 8292               | 459.6 ± 44.9**                  |
| Control group<br>(high-fat diet administration group) | 469.1 ± 54.3                    |
| <i>Kluyveromyces lactis</i> YIT 8263                  | 370.2 ± 51.1**                  |
| <i>Candida kefyr</i> YIT 8237                         | 368.5 ± 67.1**                  |
| <i>Kloeckera africana</i> YIT 8165                    | 399.5 ± 43.3**                  |
| Control group<br>(high-fat diet administration group) | 422.2 ± 57.2                    |
| <i>Hanseniaspora uvarum</i> YIT 8164                  | 390.8 ± 49.1**                  |
| <i>Issatchenkia orientalis</i> YIT 8266               | 348.5 ± 36.9**                  |
| <i>Pichia farinosa</i> YIT 8058                       | 331.9 ± 40.2**                  |
| Control group<br>(high-fat diet administration group) | 463.1 ± 66.9                    |
| <i>Torulaspora delblueckii</i> YIT 8114               | 380.1 ± 65.4**                  |

\*\*p<0.01

#### Example 5 Bile acid adsorption power

Test method;

For each of cholic acid (CA), taurocholic acid (TCA),

glycocholic acid (GCA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA), a sodium salt thereof (product of Sigma) was dissolved in a 0.1M phosphate buffer (pH 6.7) or a 0.1M phosphate buffer (pH 7.5) such that the final concentration was adjusted to 1 mM.

Lyophilized cells (100 mg) of *Issatchenkia orientalis* YIT 8266 were placed in a centrifugation tube (15 mL), and each bile acid solution (3.5 mL) prepared above was added thereto. The mixture was shaken (120 spm) at 37°C. One hour later, yeast cells were caused to precipitate through centrifugation (12,000 rpm, 10 minutes), to thereby collect the supernatant. The bile acid contained in the supernatant was quantitated by use of Enzabile II (product of Daiichi Pure Chemicals Co., Ltd.), and percent bile acid adsorption was calculated on the basis of the following formula. As used herein, the term "percent bile acid adsorption" refers to a ratio (%) of the amount of bile acid adsorbed by yeast to the total amount of added bile acid, and the ratio serves as an index of the adsorption strength between the yeast and the bile acid.

$$\text{percent bile acid adsorption (\%)} = \frac{[\{\text{added bile acid (nmol)} - \text{bile acid in supernatant (nmol)}\} / \text{added bile acid (nmol)}] \times 100}{}$$

Table 17 shows the percent bile acid adsorption. *Issatchenkia orientalis* YIT 8266 has been found to adsorb cholic acid, taurocholic acid, glycocholic acid, or chenodeoxycholic acid, exhibiting excellent primary bile acid

adsorption power. The percent adsorption of bile acid by yeast did not depend on the pH (6.7 and 7.2) of the reaction mixture. These results indicate that the yeast tightly binds to primary bile acid in the large intestinal tract and inhibits deconjugation and 7 $\alpha$ -dehydroxylation caused by enterobacteria, to thereby inhibit production of secondary bile acid.

In addition, the yeast tightly binds to deoxycholic acid—a type of secondary bile acid. This indicates that, in addition to inhibiting of production of secondary bile acid, the yeast also adsorbs and immobilizes formed secondary bile acid, to thereby reduce the toxicity thereof.

Table 17

Percent Bile Acid Adsorption by *Issatchenkovia orientalis* (%)

| Yeast  | CA<br>pH6.7 | CA<br>pH7.5 | TCA<br>pH7.5 | GCA<br>pH7.5 | CDCA<br>pH7.5 | DCA<br>pH7.5 |
|--|-------------|-------------|--------------|--------------|---------------|--------------|
| <i>Issatchenkovia<br/>orientalis</i><br>YIT 8266 | 40.3        | 40.2        | 29.3         | 43.8         | 68.8          | 73.7         |

Example 6

In a manner similar to that employed in Example 5, percent adsorption of bile acid by a variety of yeasts was measured. Table 18 shows the results. All of the yeasts exhibited excellent adsorption to bile acids; i.e., chenodeoxycholic acid (primary bile acid) and deoxycholic acid (secondary bile acid).

Table 18

## Percent Adsorption of Bile Acid by Yeasts

| Strains                             | YIT<br>No. | Percent<br>adsorption of<br>bile acid (%) |                |
|-------------------------------------|------------|---|----------------|
|                                     |            | CDCA                                      | DCA<br>(pH7.5) |
| <i>Kluyveromyces marxianus</i>      | 8292       | 63.0                                      | 60.0           |
| <i>Kluyveromyces lactis</i>         | 8263       | 54.4                                      | 41.0           |
| <i>Kluyveromyces thermotolerans</i> | 8294       | 79.5                                      | 53.6           |
| <i>Hanseniaspora uvarum</i>         | 8164       | 68.0                                      | 40.3           |
| <i>Saccharomyces cerevisiae</i>     | 8116       | 66.6                                      | 54.5           |
| <i>Saccharomyces dairensis</i>      | 8191       | 50.8                                      | 44.7           |
| <i>Saccharomyces exiguum</i>        | 8109       | 55.5                                      | 50.4           |
| <i>Saccharomyces unisporus</i>      | 8226       | 58.8                                      | 65.5           |
| <i>Saccharomyces bayanus</i>        | 8128       | 61.8                                      | 64.3           |
| <i>Hyphopichia burtonii</i>         | 8299       | 51.6                                      | 35.3           |
| <i>Candida kefyr</i>                | 8237       | 56.2                                      | 55.3           |
| <i>Candida etchellsii</i>           | 8278       | 55.8                                      | 45.1           |
| <i>Candida zeylanoides</i>          | 8018       | 76.1                                      | 56.0           |
| <i>Candida solani</i>               | 8023       | 52.0                                      | 36.9           |
| <i>Candida maltosa</i>              | 8283       | 58.3                                      | 46.4           |
| <i>Candida tropicalis</i>           | 8286       | 58.3                                      | 40.1           |
| <i>Candida cylindracea</i>          | 8276       | 58.8                                      | 53.0           |
| <i>Candida utilis</i>               | 8204       | 51.6                                      | 42.2           |
| <i>Torulaspora delbrueckii</i>      | 8313       | 58.6                                      | 52.6           |
| <i>Torulaspora delbrueckii</i>      | 8133       | 64.6                                      | 49.4           |
| <i>Pichia anomala</i>               | 8297       | 56.4                                      | 43.4           |
| <i>Pichia anomala</i>               | 8298       | 51.6                                      | 60.9           |
| <i>Pichia holstii</i>               | 8038       | 58.8                                      | 54.6           |
| <i>Zygosaccharomyces rouxii</i>     | 8129       | 48.0                                      | 39.8           |
| Cellulose (Comparative)             | -          | 4.1                                       | 3.7            |

Example 7 Inhibition of secondary bile acid production by lyophilized cells of *Issatchenkovia orientalis* YIT 8266

### Preliminary test

Male, 5-week-old Wistar rats (obtained from Nihon Kurea) had been bred for 7 days with diet powder F-2 (product of Oriental Yeast). The rats were divided into groups such that no weight difference generates among the groups, each group containing 8 rats. To each group, the diet shown in Table 19 was administered for 14 days. The rats were bred individually in a metal-made cage, and allowed to consume diet and water *ad libitum*.

Table 19

| Ingredient          | Diet Composition [%] |                     |
|---------------------|----------------------|---------------------|
|                     | Ordinary diet        | High-bile-acid diet |
| Casein              | 22.30                | 22.30               |
| Sucrose             | 56.55                | 55.80               |
| Inorganic salt      | 4.00                 | 4.00                |
| Vitamins            | 1.00                 | 1.00                |
| Soybean oil         | 1.00                 | 1.00                |
| Lard                | 10.00                | 10.00               |
| Choline bitartrate  | 0.15                 | 0.15                |
| Cholesterol         | 0.00                 | 0.50                |
| Sodium cholate      | 0.00                 | 0.25                |
| Filter paper powder | 5.00                 | 5.00                |

After completion of 14 days' breeding, no significant differences in diet consumption and in body weight increase were observed between the ordinary-diet administration group and the high-bile-acid-diet administration group.

### Bile acid level

Lyophylized feces (day 12 to day 14) (25 mg) were weighed and placed in a test tube (15 mL) equipped with a screw cap, and an internal standard substance (5- $\beta$ -pregnane-

$3\alpha$ ,  $17\alpha$ ,  $20\alpha$ -triol) (0.15  $\mu\text{mol}$ ) and ethanol (5 mL) were added thereto. The mixture was heated at 70°C for two hours by means of a block heater. The resultant insoluble matter was precipitated through centrifugation (3,000 rpm, 15 minutes). The supernatant was transferred to another test tube and dried to solid under nitrogen flow. The extract was dissolved in methanol (0.5 mL), and the resultant insoluble matter was removed by means of a filter (0.45  $\mu\text{m}$ ) (product of Nihon Millipore Ltd.). An analysis sample (10  $\mu\text{L}$ ) was subjected to separation by means of an HPLC system (product of Jasco) under the following conditions.

Table 20

Conditions for Separating Bile Acids

|                        |  |
|------------------------|--|
| Liquid A:              | Acetonitrile / Methanol / 30 mM Ammonium acetate<br>(30:30:40) mixture   |
| Liquid B:              | Acetonitrile / Methanol / 30 mM Ammonium acetate<br>(20:20:60) mixture   |
| Elution<br>conditions: | Proportional concentration gradient from 100% B +<br>0% A to 0% B + 100% A over 32 min. followed by<br>elution for 13 min. by use of 100% A. |
| Flow rate:             | 1.0 mL/min   |
| Column<br>temperature: | 25°C   |

Bilepak-II (4.6 mm i.d.  $\times$  250 mm) (separation column, product of Jasco)

The eluate provided from a separation column was mixed, at a flow rate of 1.0 mL/minute, with a reaction mixture (0.3 mM  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NAD $^+$ ), 1 mM ethylenediaminetetraacetic acid, and 10 mM potassium phosphate buffer (pH 7.8) containing 0.05% 2-mercaptoethanol).

The liquid mixture was transferred to an enzyme column (Enzymepak (4.6 mm i.d. × 35 mm, product of Jasco) charged with 3 $\alpha$ -hydroxysteroid dehydrogenase, where NADH (reduced form of  $\beta$ -NAD $^+$ ) produced upon dehydroxylation of bile acid was monitored by means of a fluorescence detector (excitation wavelength 345 nm, emission wavelength 470 nm).

Each bile acid was identified on the basis of the retention time with reference to the standard. The bile acid concentration was obtained from the corresponding peak area. In this case, percent recovery was corrected by the peak area of the internal standard.

Table 21 shows the results. The results indicate that administration of cholic acid increases the level of primary bile acid reaching the large intestinal tract, to thereby promote production of secondary bile acid. Thus, the effect of a secondary bile acid production inhibitor can be evaluated at high sensitivity.

Table 21

Level of Bile Acids Excreted in Feces [ $\mu\text{mol}/\text{day}$ ]

|                            | Ordinary diet   | High-bile-acid diet             |
|----------------------------|-----------------|---------------------------------|
| <b>Primary bile acid</b>   |                 |                                 |
| $\alpha$ -Muricholic acid  | 1.52 $\pm$ 0.35 | 8.52 $\pm$ 3.05**               |
| $\beta$ -Muricholic acid   | 0.20 $\pm$ 0.09 | 8.48 $\pm$ 1.55**               |
| Hyocholic acid             | 0.00 $\pm$ 0.00 | 0.23 $\pm$ 0.21*                |
| Cholic acid                | 0.00 $\pm$ 0.00 | 29.20 $\pm$ 7.44**              |
| Chenodeoxycholic acid      | 0.00 $\pm$ 0.00 | 0.57 $\pm$ 0.20**               |
| Total primary bile acids   | 1.72 $\pm$ 0.40 | 47.00 $\pm$ 10.31**             |
| <b>Secondary bile acid</b> |                 |                                 |
| Deoxycholic acid           | 0.72 $\pm$ 0.26 | 9.13 $\pm$ 5.92**               |
| Lithocholic acid           | 0.18 $\pm$ 0.10 | 0.13 $\pm$ 0.18 <sup>n.s.</sup> |
| Hyodeoxycholic acid        | 0.01 $\pm$ 0.03 | 1.06 $\pm$ 0.54*                |
| Total secondary bile acids | 0.90 $\pm$ 0.35 | 10.32 $\pm$ 5.84**              |

Note: Student's t-test

\* Significance level 5%

\*\* Significance level 1%

n.s. no significance

Inhibition of secondary bile acid production by lyophilized cells of *Issatchenkia orientalis* YIT 8266

Lyophylized cells of *Issatchenkia orientalis* YIT 8266 were added to a high-bile-acid diet, and the resultant diet was administered for 14 days (5% mix diet) (Table 22). The energy score of each diet was equalized by controlling the amount of casein, saccharose, lipid, or cellulose. Administration of yeast cells provided substantially no influence on diet consumption and substantially no influence

on increase in body weight during the test period (Table 23).

Table 22

Diet Composition [%]

| Ingredients                | High-bile-acid diet | Yeast-added<br>high-bile-acid<br>diet |
|----------------------------|---------------------|---------------------------------------|
| Casein                     | 22.30               | 20.56                                 |
| Sucrose                    | 55.80               | 54.15                                 |
| Inorganic salt             | 4.00                | 4.00                                  |
| Vitamins                   | 1.00                | 1.00                                  |
| Soybean oil                | 1.00                | 1.00                                  |
| Lard                       | 10.00               | 9.85                                  |
| Choline<br>bitartrate      | 0.15                | 0.15                                  |
| Cholesterol                | 0.50                | 0.50                                  |
| Sodium cholate             | 0.25                | 0.25                                  |
| Filter paper               | 5.00                | 3.55                                  |
| Powder                     |                     |                                       |
| Heat-dried<br>yeast cells* | 0.00                | 5.00                                  |

\**Issatchenkia orientalis* YIT 8266

Table 23

Diet Consumption and Body Weight Increase  
during Test Period

|                                     | High-bile-acid<br>diet | Yeast-added<br>high-bile-acid<br>diet |
|-------------------------------------|------------------------|---------------------------------------|
| Diet consumption<br>[g/14 days]     | 236.0 ± 8.6            | 227.8 ± 21.5 <sup>n.s.</sup>          |
| Body weight<br>increase [g/14 days] | 95.9 ± 4.0             | 93.6 ± 11.9 <sup>n.s.</sup>           |

Note: Student's t-test

n.s. no significance

As shown in Tables 24 and 25, through administration of

*Issatchenkovia orientalis* YIT 8266, the level of secondary bile acid excreted in feces decreased by approximately 82%, and the secondary bile acid concentration in feces decreased by approximately 84%. This tendency was observed to be particularly remarkable for deoxycholic acid and lithocholic acid. The level of primary bile acid excreted in feces and the primary bile acid concentration in feces were significantly increased through administration of the corresponding cells.

Thus, *Issatchenkovia orientalis* YIT 8266 exerted an effect of strongly inhibiting conversion of primary bile acid to secondary bile acid.

Table 24

Level of Bile Acids Excreted in Feces [ $\mu\text{mol}/\text{day}$ ]

|                               | High-bile-acid<br>diet | Yeast-added<br>high-bile-acid<br>diet |
|-------------------------------|------------------------|---------------------------------------|
| <u>Primary bile acid</u>      |                        |                                       |
| $\alpha$ -Muricholic acid     | 13.70 $\pm$ 3.40       | 15.27 $\pm$ 3.12 <sup>n.s.</sup>      |
| $\beta$ -Muricholic acid      | 7.32 $\pm$ 2.07        | 11.60 $\pm$ 2.48 <sup>**</sup>        |
| Hyocholic acid                | 0.12 $\pm$ 0.17        | 0.29 $\pm$ 0.15 <sup>n.s.</sup>       |
| Cholic acid                   | 24.37 $\pm$ 5.86       | 35.30 $\pm$ 2.55 <sup>**</sup>        |
| Chenodeoxycholic acid         | 1.10 $\pm$ 0.41        | 1.34 $\pm$ 0.27 <sup>n.s.</sup>       |
| Total primary<br>Bile acids   | 46.60 $\pm$ 7.30       | 63.80 $\pm$ 6.81 <sup>**</sup>        |
| <u>Secondary bile acid</u>    |                        |                                       |
| Deoxycholic acid              | 10.10 $\pm$ 6.51       | 0.22 $\pm$ 0.46 <sup>**</sup>         |
| Lithocholic acid              | 0.35 $\pm$ 0.31        | 0.00 $\pm$ 0.00 <sup>*</sup>          |
| Hyodeoxycholic acid           | 0.80 $\pm$ 0.32        | 1.81 $\pm$ 0.64 <sup>n.s.</sup>       |
| Total secondary<br>Bile acids | 11.25 $\pm$ 6.59       | 2.03 $\pm$ 0.81 <sup>**</sup>         |

Note: Student's t-test

\* Significance level 5%

\*\* Significance level 1%

n.s. no significance

Table 25

## Composition of Bile Acids in Feces [%]

|                               | High-bile-acid<br>diet | Yeast-added<br>high-bile-acid<br>diet |
|-------------------------------|------------------------|---------------------------------------|
| <u>Primary bile acid</u>      |                        |                                       |
| $\alpha$ -Muricholic acid     | 23.58 ± 5.32           | 23.11 ± 3.47 <sup>n.s.</sup>          |
| $\beta$ -Muricholic acid      | 12.59 ± 3.07           | 17.50 ± 2.51**                        |
| Hyocholic acid                | 0.19 ± 0.27            | 0.43 ± 0.22 <sup>n.s.</sup>           |
| Cholic acid                   | 42.47 ± 11.42          | 53.87 ± 3.01*                         |
| Chenodeoxycholic acid         | 1.89 ± 0.65            | 2.06 ± 0.50 <sup>n.s.</sup>           |
| Total primary<br>bile acids   | 80.72 ± 11.55          | 96.97 ± 0.98**                        |
| <u>Secondary bile acid</u>    |                        |                                       |
| Deoxycholic acid              | 17.30 ± 11.46          | 0.30 ± 0.67**                         |
| Lithocholic acid              | 0.59 ± 0.53            | 0.00 ± 0.00*                          |
| Hyodeoxycholic acid           | 1.39 ± 0.59            | 2.73 ± 0.89**                         |
| Total secondary<br>bile acids | 19.28 ± 11.55          | 3.03 ± 0.98**                         |
|                               | High-bile-acid<br>diet | Yeast-added<br>high-bile-acid<br>diet |

Note: Student's t-test

\* Significance level 5%

\*\* Significance level 1%

n.s. no significance

Lowering pH in the cecum by lyophilized cells of  
*Issatchenkovia orientalis* YIT 8266

The contents in the decum (day 14) (0.5 g) were suspended in purified water (4.5 mL), and a 10% aqueous perchloric acid solution (0.5 mL) was added to the suspension. The resultant suspension was stored at 4°C overnight. The suspension was centrifuged (9,000 rpm, 10 minutes), and the

supernatant was collected and passed through a filter (0.45 µm) (product of Nihon Millipore Ltd.). The thus-prepared analysis sample was subjected to HPLC.

Short-chain fatty acids were analyzed by means of an HPLC system (product of Toa Denpa Kogyo) under the following conditions. The pH buffer was mixed with the eluent immediately before passage of the detector.

Table 26

Short-Chain Fatty Acid Analysis Conditions

|                      |  |
|----------------------|--|
| Column:              | KC-811 Shodex (Showa Denko) × 2  |
| Eluent (flow):       | 15mM Perchloric acid solution containing 7% acetonitrile (1 mL/min)  |
| Column temperature:  | 42°C   |
| pH-Regulator (flow): | 15 mM Perchloric acid solution containing 7% acetonitrile and 60 mM tris(hydroxymethyl)aminomethane (1 mL/min) |
| Detector:            | Conductivity detector (Toa Denpa Kogyo)  |
| Cell temperature:    | 45°C   |

Table 27

## pH and Short-Chain Fatty Acid Content in the Cecum

|                                  | High-bile-acid<br>diet | Yeast-added<br>high-bile-acid<br>diet |
|----------------------------------|------------------------|---------------------------------------|
| pH in the cecum                  | 6.65 ± 0.08            | 6.46 ± 0.20*                          |
| <u>Short-Chain Fatty Acid</u>    |                        |                                       |
| <u>content [mM]</u>              |                        |                                       |
| Acetic acid                      | 26.6 ± 8.4             | 37.5 ± 4.0**                          |
| Propionic acid                   | 7.2 ± 2.3              | 17.5 ± 4.0**                          |
| Butyric acid                     | 2.2 ± 1.4              | 3.9 ± 0.8*                            |
| Lactic acid                      | 2.5 ± 1.0              | 2.6 ± 1.2 <sup>n.s.</sup>             |
| Succinic acid                    | 15.5 ± 7.3             | 21.8 ± 7.4 <sup>n.s.</sup>            |
| Formic acid                      | 1.1 ± 0.6              | 1.8 ± 1.3 <sup>n.s.</sup>             |
| Isobutyric acid                  | 0.7 ± 0.6              | 0.4 ± 0.4 <sup>n.s.</sup>             |
| Total short-chain<br>fatty acids | 55.9 ± 15.7            | 85.6 ± 5.2**                          |

Note: Student's t-test

\* Significance level 5%

\*\* Significance level 1%

n.s. no significance

High-bile-acid-diet groups exhibited a pH in the cecum of 6.65 and a total amount of short-chain fatty acid of 55.9 mM, whereas yeast (*Issatchenkovia orientalis* YIT 8266)-added high-bile-acid-diet groups exhibited a pH in the cecum of 6.46 (significance level 5%) and a total amount of short-chain fatty acid of 85.6 mM (significance level 1%), indicating decrease in pH in the cecum. Thus, the large intestinal pH condition was shifted to more acidic, to thereby deactivate 7α-dehydroxylase of enterobacteria.

Inhibition of cholesterol metabolism by lyophilized cells of *Issatchenkovia orientalis* YIT 8266

Feces (day 14) (25 mg) were weighed and placed in a

test tube (15 mL) equipped with a screw cap, and an internal standard substance ( $5\beta$ -cholestane) (0.1  $\mu$ mol) and a 90% ethanol solution containing 1N sodium hydroxide (1 mL) were added thereto. The mixture was heated at 80°C by means of a block heater. One hour after, distilled water (0.5 mL) was added, and neutral sterols were taken through two rounds of extraction by use of petroleum ether (2.5 mL). The resultant extract was washed with distilled water (7.5 mL), dehydrated over sodium sulfate, and dried to solid under nitrogen gas flow. The recovered neutral sterols were trimethylsilylated, and analyzed through gas chromatography under the following conditions (Table 28).

Table 28

Chromatographic (GC) Analysis Conditions

|                     |  |
|---------------------|--|
| GC apparatus:       | Model 5890 (Yokogawa-Hewlett-Packard)  |
| Column:             | SPB-1 FS-coated capillary glass column<br>(Supelco) 0.32 mm i.d. $\times$ 30 m |
| Column temperature: | 250°C, 8 min $\rightarrow$ 270°C, 10°C/min $\rightarrow$ 270°C,<br>15 min      |
| Carrier (flow):     | He (0.8 mL/min)  |
| Injector:           | Spilit, T = 230°C  |
| Detector:           | FID, T = 280°C   |
| Sample injection:   | 1 $\mu$ L  |
| Analysis time:      | 25 min   |

Through administration of *Issatchenkovia orientalis* YIT 8266, the concentrations in feces of coprostanol and coprostanone—decreased cholesterol metabolites in the intestine significantly (Table 29). Since these cholesterol metabolites in the large intestine are related to onset of diseases such as colorectal cancer (Suzuki, K., et al.,

*Cancer Lett.*, 33, 307-316, 1986), *Issatchenka orientalis* YIT 8266 is thought to be capable of preventing or treating a variety of diseases, by means of inhibiting production of cholesterol metabolites.

Table 29

Neutral Cholesterol Composition in Feces [%]

|                            | High-bile-acid diet | Yeast-added high-bile-acid diet |
|----------------------------|---------------------|---------------------------------|
| Coprostanol                | 25.7 ± 12.5         | 9.2 ± 11.9*                     |
| Coprostanone               | 1.9 ± 2.5           | 0.4 ± 0.6 <sup>n.s.</sup>       |
| Cholesterol                | 72.3 ± 14.2         | 90.5 ± 12.4*                    |
| Coprostanol + Coprostanone | 27.7 ± 14.2         | 9.5 ± 12.4*                     |

Note: Student's t-test

\* Significance level 5%

n.s. no significance

Example 8 Inhibition of secondary bile acid production by cell contents of *Issatchenka orientalis* YIT 8266

Preparation of cell contents

Lyophilized cells of *Issatchenka orientalis* YIT 8266 were suspended in a 0.1M phosphate buffer (pH 8.5), and the cells were mechanically ground by means of DYNOMILL (product of Shinmaru Enterprises). During grinding, 0.45 mm glass beads were employed as a grinding agent, and grinding operation was repeated until grinding of 95% or more cells was confirmed through microscopic observation. The milled cell liquid was centrifuged (9,000 g, 15 minutes), to thereby isolate cell contents in the supernatant. The cell contents were collected and lyophilized.

Inhibition of secondary bile acid production by cell contents  
of *Issatchenkia orientalis* YIT 8266

In a manner similar to that employed in Example 7, the effect of the cell contents prepared from *Issatchenkia orientalis* YIT 8266 on inhibition of secondary bile acid production was confirmed.

Table 30 shows the diet composition employed in the test. Administration of yeast cell contents exerted substantially no influence on diet consumption and substantially no influence on increase in body weight during the test period (Table 31).

Table 30

| Ingredients         | Diet Composition (%) |  |
|---------------------|----------------------|--|
|                     | High-bile-acid diet  | Cell-content-added high-bile-acid diet |
| Casein              | 22.30                | 20.78                                  |
| Sucrose             | 55.80                | 53.12                                  |
| Inorganic salt      | 4.00                 | 4.00                                   |
| Vitamins            | 1.00                 | 1.00                                   |
| Soybean oil         | 1.00                 | 1.00                                   |
| Lard                | 10.00                | 9.77                                   |
| Choline bitartrate  | 0.15                 | 0.15                                   |
| Cholesterol         | 0.50                 | 0.50                                   |
| Sodium cholate      | 0.25                 | 0.25                                   |
| Filter paper powder | 5.00                 | 4.43                                   |
| Cell contents       | 0.00                 | 5.00                                   |

Table 31

Diet Consumption and Increase in Body Weight  
during the Test Period

|                                     | High-bile-acid<br>diet | Cell-content-added<br>high-bile-acid<br>diet |
|-------------------------------------|------------------------|--|
| Diet consumption<br>[g/14 days]     | $232.8 \pm 9.9$        | $220.7 \pm 6.5^{\text{n.s.}}$                |
| Body weight increase<br>[g/14 days] | $95.9 \pm 4.0$         | $88.4 \pm 4.5^{\text{n.s.}}$                 |

Note: Student's t-test

n.s. no significance

As shown in Tables 32 and 33, as a result administration of cell contents of *Issatchenkovia orientalis* YIT 8266, the level of secondary bile acid excreted in feces decreased by approximately 76%, and the secondary bile acid concentration in feces decreased by approximately 70%.

Thus, similar to the case of lyophilized *Issatchenkovia orientalis* YIT 8266, cell contents thereof also exerted an effect of strongly inhibiting conversion of primary bile acid to secondary bile acid.

Table 32

Level of Bile Acids Excreted in Feces [ $\mu\text{mol}/\text{day}$ ]

|                               | High-bile-acid<br>diet | Cell-content-added<br>high-bile-acid<br>diet |
|-------------------------------|------------------------|--|
| <u>Primary bile acid</u>      |                        |  |
| $\alpha$ -Muricholic acid     | 8.56 $\pm$ 1.85        | 6.80 $\pm$ 2.04 <sup>n.s.</sup>              |
| $\beta$ -Muricholic acid      | 6.80 $\pm$ 2.18        | 8.70 $\pm$ 3.04 <sup>n.s.</sup>              |
| Hyocholic acid                | 0.20 $\pm$ 0.15        | 0.25 $\pm$ 0.06 <sup>n.s.</sup>              |
| Cholic acid                   | 28.96 $\pm$ 11.99      | 29.99 $\pm$ 4.77 <sup>n.s.</sup>             |
| Chenodeoxycholic acid         | 0.44 $\pm$ 0.29        | 0.64 $\pm$ 0.35 <sup>n.s.</sup>              |
| Total primary<br>bile acids   | 44.96 $\pm$ 13.17      | 46.37 $\pm$ 9.40 <sup>n.s</sup>              |
| <u>Secondary bile acid</u>    |                        |  |
| Deoxycholic acid              | 9.87 $\pm$ 5.46        | 2.01 $\pm$ 2.26 <sup>**</sup>                |
| Lithocholic acid              | 0.02 $\pm$ 0.05        | 0.00 $\pm$ 0.00 <sup>n.s.</sup>              |
| Hyodeoxycholic acid           | 0.65 $\pm$ 0.36        | 0.49 $\pm$ 0.14 <sup>n.s.</sup>              |
| Total secondary<br>bile acids | 10.54 $\pm$ 5.29       | 2.51 $\pm$ 2.18 <sup>**</sup>                |

Note: Student's t-test

\*\* Significance level 1%

n.s. no significance

Table 33

## Composition of Bile Acids in Feces [%]

|                               | High-bile-acid<br>diet | Cell-content-added<br>high-bile-acid<br>diet |
|-------------------------------|------------------------|--|
| <u>Primary bile acid</u>      |                        |  |
| $\alpha$ -Muricholic acid     | 16.01 ± 4.33           | 13.75 ± 1.82 <sup>n.s.</sup>                 |
| $\beta$ -Muricholic acid      | 12.39 ± 3.19           | 17.30 ± 3.29 <sup>**</sup>                   |
| Hyocholic acid                | 0.34 ± 0.20            | 0.51 ± 0.09 <sup>n.s.</sup>                  |
| Cholic acid                   | 51.04 ± 10.63          | 62.47 ± 6.95 <sup>**</sup>                   |
| Chenodeoxycholic acid         | 0.76 ± 0.34            | 1.27 ± 0.56 <sup>n.s.</sup>                  |
| Total primary<br>bile acids   | 79.82 ± 8.31           | 94.02 ± 3.61 <sup>**</sup>                   |
| <u>Secondary bile acid</u>    |                        |  |
| Deoxycholic acid              | 8.29 ± 8.90            | 3.64 ± 3.76 <sup>**</sup>                    |
| Lithocholic acid              | 0.04 ± 0.11            | 0.00 ± 0.00 <sup>n.s.</sup>                  |
| Hyodeoxycholic acid           | 1.13 ± 0.38            | 1.07 ± 0.44 <sup>n.s.</sup>                  |
| Total secondary<br>bile acids | 20.18 ± 8.31           | 5.98 ± 3.61 <sup>**</sup>                    |

Note: Student's t-test

\*\* Significance level 1%

n.s. no significance

In terms of the total amount of short-chain fatty acid and the pH in the cecum, high-bile-acid-diet groups exhibited a total amount of 54.0 mM and a pH of 6.65, whereas yeast-cell contents-added high-bile-acid-diet groups exhibited a total amount of 68.3 mM (significance level 5%) and a pH of 6.46 (significance level 5%). Thus, the yeast cell contents shifted the large intestinal pH condition to more acidic by increasing the amount of short-chain fatty acid, to thereby deactivate 7 $\alpha$ -dehydroxylase of enterobacteria.

Table 34

pH and Short-Chain Fatty Acid Content in the Cecum

|                                  | High-bile-acid<br>diet | Cell-content-added<br>high-bile-acid<br>diet |
|----------------------------------|------------------------|--|
| pH in the cecum                  | 6.65 ± 0.08            | 6.46 ± 0.20*                                 |
| <u>Short-Chain Fatty Acid</u>    |                        |  |
| <u>content [mM]</u>              |                        |  |
| Acetic acid                      | 26.2 ± 9.0             | 30.2 ± 7.1 <sup>n.s.</sup>                   |
| Propionic acid                   | 7.2 ± 2.5              | 14.3 ± 2.2**                                 |
| Butyric acid                     | 2.2 ± 1.5              | 2.1 ± 0.9 <sup>n.s.</sup>                    |
| Lactic acid                      | 2.4 ± 1.0              | 1.9 ± 1.2 <sup>n.s.</sup>                    |
| Succinic acid                    | 14.3 ± 7.1             | 17.8 ± 6.4 <sup>n.s.</sup>                   |
| Formic acid                      | 0.9 ± 0.3              | 1.8 ± 1.7 <sup>n.s.</sup>                    |
| Isobutyric acid                  | 0.7 ± 0.7              | 0.2 ± 0.2 <sup>n.s.</sup>                    |
| Total short-chain<br>fatty acids | 54.0 ± 15.9            | 68.3 ± 13.9*                                 |

Note: Student's t-test

\* Significance level 5%

\*\* Significance level 1%

n.s. no significance

As a result of administration of cell contents of *Issatchenkovia orientalis* YIT 8266, the concentrations in feces of coprostanol and coprostanone—decreased cholesterol metabolites in the intestine significantly. Thus, similar to the case of the lyophilized cells, cell contents of *Issatchenkovia orientalis* YIT 8266 are thought to be capable of preventing or treating a variety of diseases by inhibiting production of cholesterol metabolites.

Table 35

## Neutral Sterol Composition in Feces [%]

|                            | High-bile-acid diet | Cell-content-added high-bile-acid diet |
|----------------------------|---------------------|--|
| Coprostanol                | 21.1 ± 8.2          | 10.0 ± 9.9*                            |
| Coprostanone               | 2.0 ± 1.6           | 0.5 ± 0.5**                            |
| Cholesterol                | 76.9 ± 9.6          | 89.6 ± 10.2*                           |
| Coprostanol + Coprostanone | 23.1 ± 9.6          | 12.1 ± 10.7*                           |

Note: Student's t-test

\* Significance level 5%

\*\* Significance level 1%

Example 9

By use of a variety of yeast cells employed in the aforementioned Examples, foods and drinks of the following compositions were produced.

## 1) Food for maintaining and promoting health (tablets)

A composition containing the following additives was pelletized, to thereby prepare tablets.

Table 36

## Composition of Food for Maintaining and Promoting Health [%]

|                         |    |
|-------------------------|----|
| Dried yeast cells       | 10 |
| Plant extract powder    | 30 |
| Royal jelly powder      | 5  |
| Collagen powder         | 5  |
| Lactose                 | 25 |
| Corn starch             | 20 |
| Hydroxypropyl cellulose | 4  |
| Mg stearate             | 1  |

2) Drink for maintaining and promoting health

A drink for maintaining and promoting health was produced in accordance with the following formulation.

Table 37

Composition of Drink for Maintaining and Promoting Health [%]

|                          |                 |
|--------------------------|-----------------|
| Dried yeast cells        | 5               |
| Honey                    | 15              |
| Citric acid              | 0.1             |
| dl-Malic acid            | 0.1             |
| Plant extract (cinnamon) | 20              |
| D-Sorbitol liquid (70%)  | 10              |
| Sodium benzoate          | 0.05            |
| Flavors                  | suitable amount |
| Purified water           | balance         |

3) Fruit-juice-blended drink

The drink was produced in accordance with the following formulation.

Table 38

Composition of Fruit-Juice-Blended Drink [%]

|                      |                 |
|----------------------|-----------------|
| Glucose liquid sugar | 33              |
| Grapefruit juice     | 60              |
| Dried yeast cells    | 5               |
| Flavors              | suitable amount |
| Sour agent           | suitable amount |

4) Fermented milk

Fermented milk containing heated yeast cells was produced in accordance with the following formulation.

To a sterilized mixture of 10% skimmed milk powder liquid and 5% glucose, a bacterium belonging to *Lactobacillus* was inoculated, to thereby produce yogurt. To the yogurt, heated yeast cells obtained in Example 1 were added in an

amount of 0.1-20%, to thereby produce fermented milk.

5) Milk liquor

To a sterilized mixture of 10% skimmed milk powder liquid and 5% glucose, a bacterium belonging to *Lactobacillus* and heated yeast cells obtained in Example 1 were inoculated simultaneously. The mixture was subjected to stationary culturing at 37°C for 48 hours, to thereby produce milk liquor.

#### Industrial Applicability

The cholesterol-lowering agent of the present invention comprising the aforementioned yeast cells or yeast cell contents exerts a remarkable effect of lowering plasma cholesterol and hepatic cholesterol, and therefore is very effective for preventing arteriosclerosis and other diseases caused by accumulation of cholesterol.

The secondary bile acid production inhibitor of the present invention strongly inhibits production of secondary bile acid through adsorption of bile acid, acidic-shift of pH condition in the large intestine, etc., and therefore is very effective for prevention and treatment of colorectal cancer, liver cancer, pancreatic cancer, bile duct cancer, cholelithiasis, etc.

In addition, the yeasts employed in the present invention having no pathogenicity, are considerably safe, as evidenced by these yeasts having been used for producing cheese, koumiss, wine, etc. from ancient times. Thus, the

cholesterol-lowering agent of the present invention comprising the yeast cells or yeast cell contents raises no problem when administered for a long period of time. This is confirmed by the finding that no death case has been observed among rats to which the agent had been perorally administered at a dose of 8 g/kg.

Thus, the cholesterol-lowering agent and secondary bile acid production inhibitor of the present invention can be used as a peroral pharmaceutical, and are also very effective for maintaining health and preventing arteriosclerosis, colovectal cancer, liver cancer, pancreatic cancer, bile duct cancer, cholelithiasis, etc. by causing constant consumption of foods containing the agent or inhibitor.

Claims (Amended)

[Received by International Bureau on 04.12.2000. Claims 1, 2, 8, 9, 15, and 16 have as originally filed have been amended.

The other claims remain unchanged. (3 pages)]

1. (Amended) A cholesterol-lowering agent containing, as an active ingredient, at least one yeast belonging to *Issatchenkovia*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, or *Torulaspora*.

2. (Amended) A cholesterol-lowering agent containing, as an active ingredient, at least one yeast selected from among *Issatchenkovia orientalis*, *Hanseniaspora uvarum*, *Kloeckera africana*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Pichia farinosa*, and *Torulaspora delbrueckii*.

3. Foods and drinks for reducing cholesterol containing a yeast as recited in claim 1 or 2.

4. A secondary bile acid production inhibitor containing a yeast as an active ingredient.

5. A secondary bile acid production inhibitor according to claim 4, wherein the yeast is at least one species selected from among *Issatchenkovia*, *Kluyveromyces*, *Hanseniaspora*, *Saccharomyces*, *Hyphopichia*, *Candida*, *Torulaspora*, *Pichia*, and *Zygosaccharomyces*.

6. A secondary bile acid production inhibitor according to claim 4, wherein the yeast is at least one species selected from among *Issatchenkovia orientalis*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces thermotolerans*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Saccharomyces*

*Saccharomyces dairensis*, *Saccharomyces exiguum*, *Saccharomyces unisporus*, *Saccharomyces bayanus*, *Hyphopichia burtonii*, *Candida kefyr*, *Candida etchellsii*, *Candida zeylanoides*, *Candida solani*, *Candida maltosa*, *Candida tropicalis*, *Candida cylindracea*, *Candida utilis*, *Torulaspora delbrueckii*, *Pichia anomala*, *Pichia holstii*, and *Zygosaccharomyces rouxii*.

7. A food and drink for inhibiting secondary bile acid production containing a yeast as recited in any one of claims 4 to 6.

8. (Amended) Use, in the production of a cholesterol-lowering agent, of at least one yeast belonging to *Candida*, *Issatchenkovia*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, or *Torulaspora*.

9. Use, in the production of a cholesterol-lowering agent, of at least one yeast selected from among *Issatchenkovia orientalis*, *Hanseniaspora uvarum*, *Kloeckera africana*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Pichia farinosa*, and *Torulaspora delbrueckii*.

10. Use of a yeast as recited in claim 1 or 2 in the production of a food and drink for reducing cholesterol.

11. Use of a yeast in the production of a secondary bile acid proctution inhibitor.

12. Use according to claim 11, wherein the yeast is at least one species selected from among *Issatchenkovia*, *Kluyveromyces*, *Hanseniaspora*, *Saccharomyces*, *Hyphopichia*, *Candida*, *Torulaspora*, *Pichia*, and *Zygosaccharomyces*.

13. Use of a yeast in the production of a secondary

bile acid production inhibitor according to claim 11, wherein the yeast is at least one species selected from among *Issatchenkia orientalis*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces thermotolerans*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Saccharomyces dairensis*, *Saccharomyces exiguum*, *Saccharomyces unisporus*, *Saccharomyces bayanus*, *Hyphopichia burtonii*, *Candida kefyr*, *Candida etchellsii*, *Candida zeylanoides*, *Candida solani*, *Candida maltosa*, *Candida tropicalis*, *Candida cylindracea*, *Candida utilis*, *Torulaspora delbrueckii*, *Pichia anomala*, *Pichia holstii*, *Pachiticospora transversensis*, and *Zygosaccharomyces rouxii*.

14. Use, in the production of a food or drink for inhibiting secondary bile acid production, of a yeast as recited in any one of claims 11 to 13.

15. (Amended) A treatment method for reducing cholesterol, comprising administration of at least one yeast belonging to *Issatchenkia*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, or *Torulaspora*.

16. (Amended) A treatment method for reducing cholesterol, comprising administration of at least one yeast selected from among *Issatchenkia orientalis*, *Hanseniaspora uvarum*, *Kloeckera africana*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Pichia farinosa*, and *Torulaspora delbrueckii*.

17. A treatment method for inhibiting secondary bile acid production, comprising administration of a yeast.

18. A method according to claim 17, wherein the yeast is at least one species selected from among *Issatchenkia*, *Kluyveromyces*, *Hanseniaspora*, *Saccharomyces*, *Hyphopichia*, *Candida*, *Torulaspora*, *Pichia*, and *Zygosaccharomyces*.

19. A method according to claim 17, wherein the yeast is at least one species selected from among *Issatchenkia orientalis*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces thermotolerans*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Saccharomyces dairensis*, *Saccharomyces exiguum*, *Saccharomyces unisporus*, *Saccharomyces bayanus*, *Hyphopichia burtonii*, *Candida kefyr*, *Candida etchellsii*, *Candida zeylanoides*, *Candida solani*, *Candida maltosa*, *Candida tropicalis*, *Candida cylindracea*, *Candida utilis*, *Torulaspora delbrueckii*, *Pichia anomala*, *Pichia holstii*, *Pachiticospora transversensis*, and *Zygosaccharomyces rouxii*.

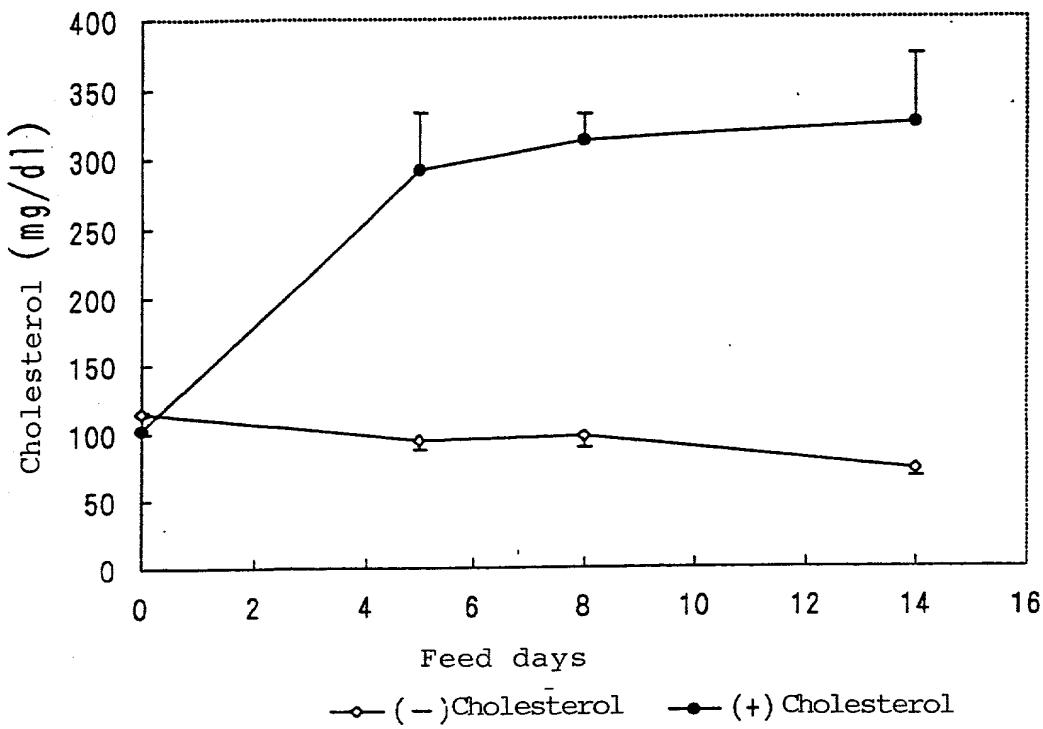
Abstract

Secondary bile acid production inhibitors and  
cholesterol-lowering agents containing a yeast as the active  
ingredient.

10/031569

OBLON ET AL (703) 413-3000  
DOCKET # 217865US SHEET 1 OF 1

Fig. 1



# Declaration and Power of Attorney For Patent Application

## 特許出願宣言書及び委任状

### Japanese Language Declaration

#### 日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者（下記の名称が複数の場合）であると信じています。

コレステロール低下剤、2次胆汁酸産生抑制剤

及び飲食品

上記発明の明細書は、

本書に添付されています。

2000年7月19日に提出され、米国特許庁または特許協定条約国際出願番号を \_\_\_\_\_ とし、

（該当する場合） \_\_\_\_\_ に訂正されました。

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

私は、連邦規則法典第37編第1条56項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

CHOLESTEROL-LOWERING AGENTS,

SECONDARY BILE ACID PRODUCTION

INHIBITORS AND FOODS AND DRINKS

the specification of which

is attached hereto.

was filed on July 19, 2000

as United States Application Number or

PCT International Application Number

PCT/JP00/04843 and was amended on  
\_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

**Japanese Language Declaration**  
(日本語宣言書)

- 私は、米国法典第35編119条 (a) - (d) 項又は365条 (b) 項に基づき下記の、米国以外の国の少なくとも一ヵ国を指定している特許協力条約365 (a) 項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

Prior Foreign Application(s)

外国での先行出願

|                                |                            |
|--------------------------------|----------------------------|
| 11-206218<br>(Number)<br>(番号)  | Japan<br>(Country)<br>(国名) |
| 2000-66659<br>(Number)<br>(番号) | Japan<br>(Country)<br>(国名) |

私は、第35編米国法典119条 (e) 項に基づいて下記の米国特許出願規定に記載された権利をここに主張いたします。

(Application No.) (Filing Date)  
(出願番号) (出願日)

私は、下記の米国法典第35編120条に基づいて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約365条 (c) に基づく権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第35編112条第1項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国提出日までの期間中に入手された、連邦規則法典第37編1条56項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

(Application No.) (Filing Date)  
(出願番号) (出願日)

(Application No.) (Filing Date)  
(出願番号) (出願日)

私は、私自信の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby claim foreign priority under Title 35, United States Code, Section 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

| Priority Claimed<br>優先権主張                       |  |
|---|--|
| 21/07/1999<br>(Day/Month/Year Filed)<br>(出願年月日) | <input checked="" type="checkbox"/> <input type="checkbox"/> |
| 10/03/2000<br>(Day/Month/Year Filed)<br>(出願年月日) | <input checked="" type="checkbox"/> <input type="checkbox"/> |
| Yes<br>はい                                       | No<br>いいえ  |
| Yes<br>はい                                       | No<br>いいえ  |

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (Filing Date)  
(出願番号) (出願日)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Status: Patented, Pending, Abandoned)  
(現況 : 特許許可済、係属中、放棄済)

(Status: Patented, Pending, Abandoned)  
(現況 : 特許許可済、係属中、放棄済)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration  
(日本語宣言書)

委任状：私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。  
(弁護士、または代理人の指名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: (*list name and registration number*)



**022850**

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|                          |   |   |  |
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(第三以降の共同発明者についても同様に記載し、署名すること)

(Supply similar information and signature for third and subsequent joint inventors.)

## Japanese Language Declaration

(日本語宣言書)

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(第六またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。)

(Supply similar information and signature for third and subsequent joint inventors.)

## Japanese Language Declaration

(日本語宣言書)

|   |  |
|---|--|
| 第七の共同発明者の氏名<br>渡辺 常一                              | Full name of seventh joint inventor, if any<br>Tunekazu WATANABE   |
| 第七の共同発明者の署名<br>渡辺常一                               | Seventh joint inventor's signature<br>Tsunekazu Watanabe   |
| 住所<br>105-8660 日本国東京都港区東新橋1丁目1番19号<br>株式会社ヤクルト本社内 | Residence C/O Kabushiki Kaisha Yakult Honsha<br>1-19, Higashishinbashi 1-chome, Minato-ku,<br>Tokyo 105-8660 JAPAN |
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|   |  |

|                   |  |
|-------------------|--|
| 第八の共同発明者の氏名       | Full name of eighth joint inventor, if any |
| 第八の共同発明者の署名<br>日付 | Eighth joint inventor's signature Date     |
| 住所                | Residence                                  |
| 国籍                | Citizenship                                |
| 郵便の宛先             | Post Office Address                        |
|                   |  |

|                   |   |
|-------------------|---|
| 第九の共同発明者の氏名       | Full name of ninth joint inventor, if any |
| 第九の共同発明者の署名<br>日付 | Ninth joint inventor's signature Date     |
| 住所                | Residence                                 |
| 国籍                | Citizenship                               |
| 郵便の宛先             | Post Office Address                       |
|                   |   |

|                   |   |
|-------------------|---|
| 第十の共同発明者の氏名       | Full name of tenth joint inventor, if any |
| 第十の共同発明者の署名<br>日付 | Tenth joint inventor's signature Date     |
| 住所                | Residence                                 |
| 国籍                | Citizenship                               |
| 郵便の宛先             | Post Office Address                       |
|                   |   |